Interactive, user-directed, computer-assisted HPLC methods development

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Abstract
An interactive, computer-assisted approach for determining multi-segment gradient elution profiles for High Performance Liquid Chromatography (HPLC) is presented. The approach is based on determining the gradient segment necessary to elute each solute at a user specified retention time. There are five main functions of this approach: (1) To inform the user of the possible retention time ranges for the solute that is going to be eluted for each elution step under both isocratic and gradient elution conditions; (2) To determine the required elution conditions, based on the user’s desired retention times and the gradient elution shape; (3) To provide the user with a simulated chromatogram; (4) To calculate the required elution profile to provide for the 'zone compression'; and (5) To determine simplified multi-segment elution profiles.

The experimental and predicted separations using this approach were found to be in good agreement.

Keywords
Chemistry, Analytical
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Interactive, user-directed, computer-assisted HPLC methods development

Qi, Fang-Fang, Ph.D.
University of New Hampshire, 1991
INTERACTIVE, USER-DIRECTED, COMPUTER-ASSISTED HPLC METHODS DEVELOPMENT

BY

FANG FANG QI
B.S., Beijing Teacher's College, 1982

DISSERTATION

Submitted to the University of New Hampshire in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Chemistry

December 1991
This dissertation has been examined and approved.

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August 20, 1991
Date
DEDICATION

This work is dedicated to my parents, Ke Chang Qi and Kun Liu, whose love and encouragement made this all possible.
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ABSTRACT

INTERACTIVE, USER-DIRECTED, COMPUTER-ASSISTED HPLC METHODS DEVELOPMENT

by

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University of New Hampshire, December 1991

An interactive, computer-assisted approach for determining multi-segment gradient elution profiles for High Performance Liquid Chromatography (HPLC) is presented. The approach is based on determining the gradient segment necessary to elute each solute at a user specified retention time. There are five main functions of this approach:

1. To inform the user of the possible retention time ranges for the solute that is going to be eluted for each elution step under both isocratic and gradient elution conditions;

2. To determine the required elution conditions, based on the user's desired retention times and the gradient elution shape;

3. To provide the user with a simulated chromatogram;

4. To calculate the required elution profile to provide for
the 'zone compression'; and

5. To determine simplified multi-segment elution profiles.

The experimental and predicted separations using this approach were found to be in good agreement.
CHAPTER 1

GENERAL INTRODUCTION

High-performance liquid chromatography (HPLC) has been practiced for approximately twenty years (1). Its development has proceeded rapidly, and it is now accepted as one of the most reliable and versatile techniques for separating mixtures of liquid compounds.

The purpose of a chromatographic analysis is to separate a mixture into individual components for determining qualitative and/or quantitative information. One study estimated that less than 5 percent of the nine million known organic compounds can be analyzed by gas chromatography (4). Chemical derivatization can be used with 10 to 20 percent of the remaining compounds (4). In contrast, HPLC is amenable for use with nonvolatile, thermally fragile materials (2,3), which include 60 to 70 percent of all known compounds. For example, HPLC can be used to separate polar and nonpolar compounds as well as biological macromolecules, such as proteins, nucleic acids and peptides.
Because of its high efficiency and reliability, HPLC has been widely used in chemistry, biochemistry and the environmental sciences. There is a wide variety of commercially available instrumentation that fully meet the requirements of different types of separation problems, from routine analysis to preparative separation.

HPLC methods development is one of the most time-consuming and often frustrating jobs in the analytical laboratory, for the following reasons:

1. Samples are often complex mixtures which are difficult to separate. This means that a number of trial-and-error runs are usually necessary before an adequate separation can be obtained.

2. Complex samples of this kind often require relatively long times (an hour or more) to achieve an adequate separation. This adds up to a substantial effort at methods development.

3. There is a large number of variables that must be taken into consideration during the methods development process, for example,
   a) mobile phase composition
   b) the duration time for a certain solvent concentration
   c) the gradient shape (i.e., step isocratic elution, linear, or complex gradient elution)
   d) flow rate
e) column conditions (stationary phase type, length, temperature, etc.).

The typical approach to HPLC methods development is still based on trial and error, relying on the experience and intuition of the chromatographer, even though modern instrumentation is available. The methods development process begins with the selection of a stationary phase and a mobile phase. After the first results are obtained, the mobile phase composition is adjusted to affect the overall retention times of the solutes or to resolve all of the components of interest. When performed by an experienced chromatographer, this process continues in a more or less efficient manner until either the separation is satisfactory or it is decided that the mobile phase composition (or stationary phase) needs to be changed and the entire process repeated. This trial and error approach is clearly inefficient for complex samples. If the operator is not an experienced chromatographer, the inefficiency of this approach to methods development is even worse.

Strategies used for HPLC methods development are rapidly changing with the increasing use of powerful personal computers which can assist in the methods development process. Several computer-assisted retention prediction systems have been developed (5-46). These systems enable chromatographers to predict retention times
and to find usable elution conditions. A few years ago, a more efficient approach to methods development, HPLC computer simulation, was developed. Computer simulation allows the user to visually simulate HPLC separations by combining a computer-assisted retention prediction system with computer graphics (28-46).

Recently there has been much interest in the use of expert systems in analytical chemistry, specifically in their application to chromatography. Expert systems are computer programs which can perform the problem solving of a human expert (121-125). Chromatographers hope that one day someone will be able to enter a chromatographic laboratory with questions about the sample and be assisted by a computer with an expert system. Based on the questions asked the computer would determine the stationary phase, mobile phase and optimal elution conditions for the sample in a short period of time. This information would be provided to an HPLC instrument, which would conduct the separation experiment. The results would then be returned to the expert system for evaluation. In theory, if an expert system were coupled to a robot, the entire process (sample preparation, introduction, separation and analysis) could be automated. Eventually, with further development, the whole process could be completed without a human expert present.
1.1 Overview of Computer-Assisted Retention Prediction

The theoretical basis for retention prediction was first given by Martin and Synge when they introduced the plate theory in 1941 (15). The plate theory was then extended to the prediction of retention times under isocratic and gradient elution conditions (16-26). From 1941 to 1957, Beukenkamp and co-workers as well as several other workers derived equations based on the plate theory which they used to calculate the minimum column height required for a desired separation (102, 106). They also calculated the elution order of solutes with stepwise changes in eluent concentration (103) so that they could determine the best conditions for a given separation (103-105). Computer-assisted retention prediction based on these theories has not enjoyed widespread use among chromatographers. The reason for the lack of use was simply that all the theories were limited in their applicability. Also, during the 1950s and 1960s computers were rarely available for use in the chromatographic laboratory.

Since the 1960s Snyder and co-workers have derived equations for retention volume and resolution in gradient and stepwise elution separations (117-119). Their approach has been successfully applied to computer-assisted retention prediction systems.

In 1970’s and early 80’s, Jandera and Churáček...
published several articles on a mathematical approach for calculating retention of solutes in isocratic, gradient and stepwise elution liquid chromatography (22, 23, 27, 109-115). Their approach permits calculation of optimum composition and volume of the mobile phase in order to achieve a desired separation. The authors pointed out that when this approach is applied the calculations should be performed with the aid of a computer.

With the advent of personal computers in the 1980s, chromatographers began using them more frequently and have been able to more easily predict retention times in HPLC. Computer simulation of HPLC experiments has developed as a more reasonable approach for HPLC methods development during the last few years (12).

1.1.1 Computer-Assisted Simulation of HPLC Experiments

Computer simulation is the combination of the general theory of retention prediction with the power of the personal computer for calculation and graphical presentation (28-46). A computer simulation system calculates retention times and band widths and presents the user with a simulated chromatogram on either the monitor or the printer.

Computer simulation is based on elementary but reliable theory pertaining to the following relationships:
dependence of retention times of solutes on mobile phase concentration;

dependence of elution band width on experimental conditions.

Computer simulation of HPLC separation allows the user to apply complex chromatographic theory to the accurate prediction of experimental results. The user simply enters the experimental conditions, such as flow rate, retention times from few experiments, and mobile phase composition, but does not need to be intimately involved in performing the detailed calculations required. Usually initial HPLC experiments must be performed. This approach requires experimental data that bracket the conditions of interest. The results of further experiments are then predicted by simulation. Computer simulation allows for the rapid "testing" of a method using the computer without the need to run large numbers of chromatographic experiments in the laboratory. Hours of laboratory work can be condensed into a few minutes resulting in saving time and laboratory supplies. Using computer simulation, the user can easily study the effect of a number of experimental variables on the separation. The initial and sequential mobile phase concentrations can be varied, the gradient times (meaning how long it takes the mobile phase composition to be changed from the initial to the final conditions) can be changed and
gradients of any shape can be explored.

For example, "Drylab", developed by L. R. Snyder and J. W. Dolan, and marketed by LC Resources Inc., is a program used for computerized simulation of chromatographic experiments. The user first enters data from two initial experimental runs into the program and then the program determines the results that would be obtained from other experiments (28-30, 32, 39, 43, 137, 138, 164-169).

Hodges and co-workers developed a computer simulation program called "Pro Digest-LC" which can predict retention behavior of peptides. Their program allows changing the sample volume, sample quantity, flow rates, gradients and desired resolution (31). Gelderloos and co-workers used a computer simulation method called "whole column detection chromatography" to predict the distribution of solutes as a function of column position at any time during the elution process (101).

1.1.2 Optimization and HPLC Methods Development by Computer-Assisted Retention Prediction

Optimization of a separation is an especially important step in any HPLC analysis. Over the years, many efforts have been made to develop systematic optimization in HPLC (50-56). Interest has focused on improving sensitivity, selectivity and separation speed. The
optimization of the separation process provides the most satisfactory resolution for all sample components of interest in as short a period of time as possible. The goals of HPLC optimization include the following (42):

1). solving the problem at minimum cost;

2). achieving separation with minimum time and effort; and

3). producing the 'best' separation possible with a given sample.

These goals do not adequately define the process. The task of optimization will depend on the problem at hand. For example, either all components may need to be separated or just some relevant components may need to be separated depending on user's needs.

Over the years, a number of optimization strategies have been developed and applied to chromatographic systems. For instance, the Simplex Optimization Procedure was first proposed in 1962 by Spendley et al. (172) and has recently become popular (57-62, 107). In principle, this procedure adjusts experimental conditions away from those which result in unfavorable responses and toward conditions which promote greater success. The great advantage of the Simplex method is that it allows the operator to optimize many variables without prior knowledge of the separation mode or the complexity of the sample. The procedure utilizes multifactors for empirical feedback, which permits rapid
identification of the optimum conditions for a separation. It is relatively efficient. However, use of this method requires a large number of experiments, typically about forty (41). The Simplex procedure may result in a local optimum, rather than the desired global optimum.

The "Window Diagram" technique of Laub and Purnell, published in 1975, has proven to be useful in finding the global optimum in situations in which a functional relationship between chromatographic retention and variables are known or can be assumed (64-69). It is a graphical method for locating areas (windows) into which all solutes may be separated. An example of the use of a window diagram to aid in the optimization of mobile phase composition in the separation of five organic compounds is shown in Figures 1-1, 1-2 and 1-3. Figure 1-1 shows experimental retention times as functions of %B (the percentage of pump B) for the five solutes. Resolution is an important parameter used in Chromatography to express separation results. The resolution is defined as the difference in retention times of two adjacent peaks divided by the average peak width at the base (108). Resolutions as a function of %B for all possible solute pairs of the five solutes can be seen in Figure 1-2. Figure 1-2 shows the areas between the axis where resolution equals zero and the lines of minimum resolution, are so-called windows. These windows are
Figure 1-1.
Retention times as functions of %B for five solutes: 1 = benzene; 2 = o-nitrotoluene; 3 = 1-bromo-4-nitrobenzene; 4 = toluene; 5 = chlorobenzene.
Figure 1-2.

Resolution as a function of %B for all possible peak pairs of the five solutes of Figure 1-1.
Figure 1-3.
Window diagram based on the results shown in Figure 1-2.
redrawn in Figure 1-3. These windows represent the areas within which separation is possible. Optimum separation can be achieved using the conditions that are indicated from the point on the %B axis which corresponds to the highest point of the highest window. This point actually represents the %B ( %B = 30% ) where the best separation between the two most difficult to separate pairs of solutes can be achieved.

The Overlapping Resolution Map (ORM) method, which was developed by Glajch et al.(63, 70), is used for the optimization of multi-solvent experiments with up to quaternary compositions. For example, in reversed-phase HPLC, methanol, acetonitrile and tetrahydrofuran can be used as modifiers. Water is generally the fourth solvent. Working with three organic modifiers, the three binary solvent compositions (water+modifier) will define the three vertices of an optimization triangle (128). Seven to ten experiments are performed, with a mobile phase intended to produce a similar capacity factor range for all components of the sample. In this method areas are located in the triangle where the resolution exceeds a certain threshold value. This is repeated for all pairs of solutes and then the results are combined to form a single plot (42).

Today, computer-assisted optimization in HPLC methods development can be done automatically with a high degree of success. There have been several successful applications of
HPLC optimization by computer simulation. Computer simulation systems, with their capacity for graphic presentation of simulated chromatograms, have been demonstrated to be particularly efficient for HPLC methods development. Visual simulation is especially helpful to chromatographers in the selection of optimum elution conditions. Snyder and co-workers have done an extensive study of the overall strategy for the optimization of a gradient elution using computer simulation as their primary approach (32).

The major steps in optimization of a HPLC method can be summarized as follows (51):
1) separation mode selection;
2) retention optimization;
3) selectivity optimization;
4) system optimization; and
5) method validation.

The methods that have been used can be classified into two categories. The first one consists of column parameters, for example: column length, column diameter, stationary phase type and particle size of the packing material. The second consists of primarily mobile phase parameters, such as solvent composition, solvent strength, pH and ionic strength.

"Separation mode selection" refers to the
determination of the appropriate separation method by HPLC (e.g., size-exclusion, partition adsorption, ion-exchange or affinity LC; normal or reversed phase LC, etc.) and the appropriate conditions (column type and size, mobile phase composition, pH, etc.). The goal of this step is to obtain chromatographic peaks for all of the sample components which are of interest.

"Retention optimization" has to do with finding the appropriate mobile phase strength. These are conditions which will bring all the retention times of the sample components into the proper time range, as decided by the user.

"Selectivity optimization" is the process of obtaining sufficient separation for all components of interest by changing either the stationary phase or the mobile phase.

"System optimization" consists of varying some of the system parameters to improve separation resolution and sensitivity and to reduce analysis time. Parameters that can be used may be the dimensions of the column, flow-rate, etc.

"Method validation" has to do with evaluating the method based on the goals of the original separation problem and the intended purpose. This is accomplished by analyzing the experimental results that were obtained in the separation.
1.1.3 Expert Systems for High Performance Liquid Chromatographic Methods Development

Expert systems are software products that either can be used to solve problems the same way a human expert would or to obtain intelligent advice on problems requiring some expertise (73). Each expert system has three basic parts: First, there is a knowledge base, which contains as much information as possible about a specific area or problem domain. The knowledge base is organized into a form that makes it accessible for solving problems. Second, there is an inference engine, the central program that manipulates the knowledge base in an effort to reach conclusions about the problems posed. Finally, there is a user interface, to enable a human expert to add to the knowledge base and to enable a novice to use the system to solve problems within a specific domain (74).

The information stored in the knowledge base may be derived from two types of sources: public theoretical knowledge and private experiential knowledge. The public knowledge of a problem domain is the information which can be found in books or journals. The private knowledge is that accumulated by human experts. It is a combination of basic theoretical knowledge and some empirically derived facts.

In HPLC, many factors affect the choice of separation
modes (reversed phase or normal phase, ion exchange, etc.), mobile phase composition, and instrumental operating conditions. In addition, requirements regarding resolution, sensitivity, and separation time vary for different samples depending on the user’s needs. Complex decisions involved in HPLC methods development are usually made by human experts, but this process is not always very efficient and human experts are not always available. Thus, in an effort to automate the HPLC methods development process, researchers have been attempting to incorporate the experience of human HPLC experts (i.e., chromatographers) into computerized expert systems (71-91). A few presentations on the topic of HPLC expert systems were published in the period from 1984 to 1988, but it is only within the past three years that most of the papers on expert systems have appeared. It is obvious that the use of expert systems in the field of chromatography is still in its early stages of development and that significant new developments are to be expected.

It is important to stress that computer simulation and automated solvent optimization systems are not expert systems. Some of the calculations they perform are, however, similar to those that would be incorporated in an HPLC expert system.

The task of an HPLC expert system is to guide chemists
in the selection of sample preparation techniques, columns (stationary phase), mobile phase constituents, and detection techniques by the use of valuable knowledge derived from human HPLC experts and from available literature. An HPLC expert system would provide users of chromatographic techniques with automated, reliable and rapid access to existing chromatographic expertise.

For a chromatographic analysis, the following modules should be included (71):

1. determination of sample preparation and detection methods;
2. selection of separation modes (stationary phase type and particle size);
3. mobile phase selection and optimization of separating conditions;
4. peak identification and on-line quantification;
5. methods validation.

A complete expert system must cover the whole process of HPLC methods development from the initial choice of sample pretreatment to the final validation of the newly developed method under a variety of separation conditions. It should assist with intermediate steps in the process, such as the choice of an optimization criterion and the optimization of the instrumental conditions.

An important step in developing an expert system is
the acquisition of a knowledge base. This is a key stage in an HPLC expert system. The basic elements of the knowledge base are rules and facts. The facts consist of the information that is generally agreed on by experts in the field. The rules consist of the processes involved in good decision making, such as logic and judgement (42).

Different expert system approaches have been used for different kinds of problems. There is no one universal approach to all expert systems.

Some of the many advantages a computerized expert system has over a human expert are that it (120):

1. can be used by many users,
2. is always available when needed (day and night),
3. provides logical explanations with every consultation, never gets tired.
4. is exactly reproducible,
5. always considers many the possibilities,
6. can suggest the optimum conditions for each separation mode, and
7. allows the use of complex equations.

Main disadvantages are (120):

1. Knowledge is fixed and limited to a small domain.
2. This system can not learn, not like human expert can improve with more experience.
1.2 Calculation of Elution Times in HPLC

A fundamental element of optimization in both computer simulation and expert systems in HPLC is the ability to calculate retention times under both isocratic and gradient elution conditions. Precise retention calculation is an important improvement in the efficient separation of complex mixtures.

In isocratic elution, the composition of the mobile phase is held constant during the elution process. The isocratic elution method is useful in the analysis of sample mixtures with a small retention range.

There are several advantages of isocratic elution over gradient elution HPLC. For example, when using isocratic elution, relatively simple instrumentation is required and re-equilibration of the column is not necessary after every HPLC run.

Gradient elution is the process of varying the composition of the mobile phase during the elution of a sample in the column. Gradient elution liquid chromatography is preferred for the separation of sample mixtures with a wide range of retention times. Isocratic separations of such mixtures usually leads either to an incomplete resolution of the early eluted solutes or to excessive elution times of sample compounds with a great affinity for the stationary phase. This is called the
Multi-segment gradient elution consists of linked isocratic or ramp steps. In this elution profile, the mobile phase composition is a function of elapsed time (or gradient time). One more variable (gradient time) makes this method flexible and powerful compared to isocratic and gradient elution. It may allow the optimization of the separation throughout the entire chromatogram.

A novel solvent delivery method, called solvent modulation, was described in 1990 by Wahl and co-workers (126, 127). In solvent modulation, individual solvent zones are introduced onto the chromatographic column in varying or repeating sequences. This is a new method with some advantages. It might become popular in the future.

1.2.1 Various Approaches for Calculating Retention Times

In the mid-1950s, Drake and Freiling derived the fundamental equation for the prediction of peak positions in gradient elution (129). Subsequently, several researchers have proposed theories for retention prediction under isocratic and gradient elution conditions (16-21, 130-131).

The theories proposed in the 1950s and 1960s, however, were limited in their applications, because early gradient liquid chromatographic hardware was simple, relying on
linear or simple curved gradients. Modern gradient HPLC instruments are typically equipped with electronic programmable devices which are capable of accurately generating linear, convex, concave or multi-step gradients.

In the past ten years, a number of approaches for calculating solute retentions for both single and multistep gradient elution experiments have been published and tested (92-97). Drouen et al. (132), Jandera and Churacek (22-23, 27, 109-115), Schoenmakers et al. (133-135), Borowko et al. (92-93), Snyder et al. (28, 30, 136-138) and others (139-143) have shown that exact mathematical equations can be derived for certain gradient elution conditions. Experiments have proven that these equations can predict the retention times of a solute with great accuracy. These approaches are generally applicable in cases where the relationship between the capacity factor $k'$ (is defined as the ratio of the amount of the solute in the stationary phase to that in the mobile phase.) and the mobile phase concentration is known or can be easily determined. The solution depends on parameters such as gradient shape, the number of components in the mobile phase and instrumental delay volume.

Tomellini and co-workers presented an approach for the calculation of solute retention under gradient elution conditions using numerical integration methods in 1985 (32).
Their approach eliminated the need for exact solutions. This approach for the calculation of solute retention can be used for any solvent composition vs. solute capacity factor relationship or solvent composition vs. time relationship problem and is, therefore, universal in nature.

1.3 Goals of This Research

The purpose of this research is to develop an approach to HPLC methods development which will provide the user with control for determining conditions for the production of desired separations. It does this by informing the user what elution times are chromatographically viable. This involves changing the strength of the mobile phase, which is a powerful, quick and easy way to control separation. The objective was to assist chromatographers in designing and developing multi-segment gradient elution profiles for HPLC experiments, decrease guesswork and also to give experienced chromatographers increased control of separations. This work provides users with a computer simulation approach for predicting results before actually performing the experiments.

This approach to methods development will allow the user to answer the following questions:
- What retention times are possible for the solutes in this sample?
- What mobile phase conditions will result in the desired retention times?
- What chromatogram will result for a given set of elution conditions?
- How can the chromatographic results be changed?
- How can a method to increase the detection sensitivity for the components of interest be designed?
- How can the gradient profile be simplified while keeping the desired retention times the same?

1.3.1 Chromatographic Equations

In HPLC, liquid mixtures are injected into the column, and then eluted by the mobile phase. In the column, the solutes distribute themselves between the stationary phase and the mobile phase. Continuous introduction of additional mobile phase forces the solutes to move through the column. Since solute movement only occurs in the mobile phase, the average velocity at which a solute migrates depends on the fraction of time it spends in the mobile phase. The different partition coefficients for the solute result in differences in speeds for them. After a certain time the solutes are separated into bands located along the length of the column, which are monitored by the detector as they exit the column.

For each solute, the time spent in the column (called
the solute's retention time, \( t_R \), is equal to the column length (\( L_{col} \)) divided by its average linear velocity (\( U_{band} \)) in the column, that is (35)

\[
L_{col} = U_{band} \times t_R
\]  

(1)

\( U_{band} \) is constant if the composition of the mobile phase is constant, as in isocratic elution. The composition of the mobile phase is varied in gradient elution, so \( U_{band} \) is generally not a constant.

The linear velocity of the mobile phase, \( u \), can be calculated by

\[
u = \frac{L_{col}}{t_M}
\]  

(2)

\( t_M \) is the dead time of the column, which is the elution time of an unretained solute. Any general calculation of retention times in either isocratic or gradient elution conditions is based on the capacity factor \( k' \). The capacity factors are dependent on the mobile phase composition.

The fundamental equation for calculating the capacity factor is:

\[
k' = \frac{t_R - t_M}{t_M} \quad \text{or} \quad t_R = t_M \left( 1 + k' \right)
\]  

(3)

where \( k' \) is the capacity factor. By substitution, equation (1) becomes

\[
L_{col} = U_{band} \times t_M \left( 1 + k' \right)
\]

or

\[
U_{band} = \frac{L_{col}}{t_M \left( 1 + k' \right)}
\]  

(4)

The distance traveled by a solute under the isocratic
elution condition, \( L_{1,\text{iso}} \), during time \( t \), can be calculated by:

\[
L_{1,\text{iso}} = L_{\text{col}} \times t / \left( t_{\text{W}} \left( 1 + k' \right) \right)
\]  

(5)

For gradient elution conditions, where \( k' \) is generally changing throughout, the instantaneous band velocity, \( U_{\text{band, inst}} \), is used to calculate the distances traveled by the solute. \( U_{\text{band, inst}} \) can be obtained from the instantaneous \( k' \), \( k'_{\text{inst}} \), by:

\[
U_{\text{band, inst}} = L_{\text{col}} / \left( t_{\text{W}} \left( 1 + k'_{\text{inst}} \right) \right)
\]  

(6)

Thus, the equation for calculating the distance traveled by the solute under gradient elution conditions becomes:

\[
L_{\text{col}} = \int_{0}^{t_{R}} L_{\text{col}} / \left( t_{\text{W}} \left( 1 + k'_{\text{inst}} \right) \right) \, dt
\]  

(7)

There are other factors which need to be considered during an elution process. It must be recognized that at the started an HPLC experiment, there is mobile phase in the column, the composition of which is the starting %B. At the beginning of a typical gradient elution experiment, the solutes start to move from the inlet of the column, but the gradient starts from the mixing chamber. There is a volume between the mixing chamber and the column. The time that it takes for the gradient front to move from the mixing chamber to the column is called the instrumental delay time \( t_{D} \). Thus, for a certain time after the injection, the solute zones will travel through the column under isocratic elution conditions, with a solvent composition identical to that
established before the beginning of the gradient. The time when the gradient mobile phase catches up with a solute is called $t_{corr}$ for that solute. During the $t_{corr}$ the distance travelled by each solute depends on the initial mobile phase composition and the relationship between its actual velocity and the mobile phase composition. The $t_{corr}$ may be different for different solutes. By this time, the solutes have travelled some distance in the column and are already located at different positions in the column. Thus, given the initial mobile phase and the gradient delay time, the distance travelled by each solute during the instrumental gradient delay can be determined. Once the distance travelled by each solute during the delay time is known, the distance which remains in the column for each solute to travel before eluting can be calculated.

For each solute, after its $t_{corr}$, when a mobile phase meets it, the distance traveled by this mobile phase is equal to

$$\left( \frac{L_{col}}{t_{m}} \right) \times t_{corr}.$$  

This distance consists of two parts. One part is the distance from the mixing chamber to the column head, which is equal to

$$\left( \frac{L_{col}}{t_{m}} \right) \times t_{D}.$$  

The second part is the distance traveled by the solute in $t_{corr}$, which is equal to
\[
\left\{ \frac{L_{c01}}{t_m (1 + k')} \right\} \times t_{\text{corr}}.
\]

Thus
\[
\left[ \frac{L_{c01}}{t_m (1 + k')} \right] \times t_{\text{corr}} + \left( \frac{L_{c01}}{t_w} \right) \times t_D = \left( \frac{L_{c01}}{t_w} \right) \times t_{\text{corr}}.
\]

Rearranging Equation (8) gives:
\[
t_{\text{corr}} = t_D \left[ \left( \frac{1}{1 + k'} \right) / k' \right].
\]

The distance traveled by each solute in \( t_{\text{corr}} \) can be obtained using:
\[
L_{iso} = \left( \frac{L_{c01} \times t_D}{t_w \times k'} \right)
\]

In Equation (10) \( k' \) is calculated from the initial isocratic condition, in which the composition of the mobile phase is the starting %B.

For multi-segment gradient elution experiments, calculations must be performed for all the elution conditions each solute encounters while in the column. When the sum of the lengths traveled by a solute equals the column length, then the sum of the time intervals equals its retention time, \( t_R \). However, a continuous correction has to be made, because the solutes and the mobile phase are both moving, although at different rates. The gradient concentrations experienced by a solute zone depend not only on the time since the gradient started but also on the position of the band in the column. Thus, the actual time spent moving and the time corresponding to the gradient concentration which the solute zone encounters are not the
same. Both a gradient time and an actual time must be calculated. If the actual time interval, \( t_{\text{actual, int}} \), is determined, the gradient time interval, \( t_{\text{grad, int}} \), can be calculated using:

\[
t_{\text{grad, int}} = t_{\text{actual, int}} - \frac{L_{\text{trav, act, int}}}{u}
\]

Where \( L_{\text{trav, act, int}} \) is the distance traveled by the solute band in the actual time interval. The sum of the gradient intervals is equal to the gradient time experienced by the solute band. This time has to be used to calculate the instantaneous capacity factors from the instantaneous concentration of the mobile phase for each of the stepwise integrations. We have chosen to use 0.01 minutes as the time interval for this work.

### 1.3.2 Procedure and Program Description

The approach developed utilizes retention time data from isocratic elution experiments for a number of mobile phase compositions. It determines the position of the solute bands in the column by calculating the velocity of the bands through the column for each 0.01 minute time interval of the gradient elution experiment.

If retention data corresponding to various proportions of the modifier for a solute in isocratic elution are available, the capacity factor under any mobile phase condition can be calculated. We have assumed that for
closed spaced data points there is a linear relationship between the capacity factor and the composition of the mobile phase between the experimentally determined points (36). Interpolation can be used for calculating any capacity factor if the concentration of the mobile phase of interest is bracketed by the experimental determined data points. The linear velocity of the band through the column under isocratic elution conditions can also be calculated in this manner.

An HPLC instrument generates a gradient elution solvent profile by increasing or decreasing the solvent composition in a series of small steps that are kept small changes in solvent composition and close to the shape of the linear gradient segment. This is essentially how gradients are produced by the HPLC instrument. Thus, a gradient can be thought of as a series of small isocratic steps, each one with a greater or a lesser percentage of the modifier depending on whether the gradient profile has a positive or a negative slope. Thus, the calculation of solute retention times for a linear gradient elution experiment is converted to the calculation of the sum of a series of discrete isocratic steps.

For a multi-segment elution profile, each segment is designed to elute one solute. A key to the approach developed is that when the solute is near the end of the
column, the gradient for eluting the next solute has to begin. Thus, when the farthest solute arrives at the end of the column, the next gradient just catches up with the solute that follows it.

Data as to column length, dead time, delay time, number of solutes, peak area for each solute and retention times as a function of the composition of the mobile phase from the isocratic elution are first entered into the program. As previously mentioned, the capacity factors for other mobile phase compositions are then calculated by linear interpolation based on the composition of the mobile phase.

The program calculates and informs the user of the possible retention time ranges for both isocratic and gradient elution experiments.

The program provides the user with five choices for each gradient elution segment:
1) Isocratic elution and no "zone compression", which will be explained in Chapter 3;
2) Isocratic elution with "zone compression";
3) Linear ramped gradient elution with no "zone compression";
4) Linear ramped gradient elution with "zone compression";
5) Simple "zone compression".

After the user enters the desired retention times and
the gradient elution shape, the program calculates the velocity required. It determines the necessary capacity factor required to achieve the desire velocity and the composition of the mobile phase which can provide that capacity factor based on the distance that the solute has to travel.

The program presents the gradient profile determined and displays a simulated chromatogram, using the predicted retention times and band widths calculated. Simulated chromatograms are given ideal Gaussian profiles with appropriate peak areas for each solute. The peak area for each solute is the same as in the isocratic elution experiment performed previously by the user.

The user can then have the elution profiles changed for different desired retention times or have the elution profile simplified.
CHAPTER 2

Interactive Methods Development for Multi-segment Gradient Elution HPLC

2.1 Introduction

Determining which elution conditions produce adequate chromatographic results, the so-called "methods development" stage of a chromatographic experiment, can be difficult and time consuming even for experts in the field. Computer-assisted simulation of chromatographic experiments is one approach to methods development (1-14). The basis for such an approach is that the scientist can simulate an experiment, using previously acquired experimental data, faster than the experiments can be performed in the laboratory. An additional benefit of chromatographic simulation is that it can provide the user with the opportunity to manipulate variables in the experiment (i.e., column length, multiple column order, etc.) which would be difficult or time consuming to vary in the laboratory.

The use of chromatographic simulation for methods development, as is generally practiced, has one significant limitation. The scientist simulating an experiment is often
only permitted to ask the same question at the computer as would be asked in the laboratory. That question is essentially: "What chromatogram will result when I use these elution conditions?". It should be noted that the information available to the computer during a simulation far exceeds that which is available to the chromatographer during an actual experiment. For example, the computer can determine at any given time during the simulated chromatographic run, the location of each solute in the column, the distance each solute must travel before eluting and the mobile phase conditions for any location in the column at any time during the experiment.

An alternate approach to the use of chromatographic simulation is to provide answers for questions which are not possible in the laboratory setting, thus freeing the scientist from many experimentally imposed limitations. For example, a fundamental advance in HPLC methods development can be made if the user is allowed to ask the question: "What elution conditions will produce the separation which I need?". One way to answer this question is to use the data available to the computer in a stepwise manner, to determine which chromatographic conditions will produce a desired chromatogram. If the program determines where the solute is in the column and how far it must travel to elute, then it can determine the solvent composition needed to
produce the velocity required for the solute to elute at the desired retention time. It should be noted, however, that this approach is limited by the chromatographic responses of the solutes and, therefore, not all desired chromatograms can be obtained. Using such an approach allows the chromatographer to better utilize the knowledge of the chromatographic experiment and conditions throughout the methods development process.

The approach to chromatographic methods development described here allows the scientist to produce desired chromatographic results by interactively designing a multistep gradient elution profile. The use of multisegment (or multistep) gradients is a logical strategy for obtaining mobile phase profiles which produce the chromatographic results desired by the scientist.

One of the most powerful ways of achieving a separation is to use a variety of mobile phase modifiers. The mobile phase composition can be varied for different separation problems. Variables include: pH, organic modifier concentration, ionic strength, etc. The pH of the mobile phase directly influences the ionization of the solutes. The reason for this is that changes in pH can change separation selectivity for ionized or ionizable solutes, since charged molecules are distributed preferentially into the aqueous or more polar phase. If the
capacity factors of the solutes depend on the pH of the mobile phase, it is reasonable to use the buffer mobile phase for a gradient elution (148-151). It may be possible to resolve a mixture of acidic and alkaline solutes by pH manipulation alone, without the use of organic modifiers.

Some experiments were performed to determine the applicability of the previously described approach for gradient elution methods development to pH gradient elutions. Since phenolic and related weak acids are important compounds in the wine and wood related industries (152-163) and their $k'$ changes significantly as a function of mobile phase pH, the separation of these compounds by HPLC pH gradient elution was chosen as the test case.

2.2 Theory and Program Description

Solute retention times are calculated based on the knowledge that each solute must travel the length of the column before eluting and that the velocity of any solute at any given time is a function of the solvent strength experienced by the solute at that time. The basic requirement for calculating the velocity of a solute is that the program must have complete access to the positions of each solute in the column and the solvent composition at any point in the column at any time during the experiment. Such information is readily available when using an approach
involving numerical integration to calculate retention times for solutes in gradient elution experiments. Programs based on this approach have been previously developed and the accuracy of calculations has been demonstrated for gradient elution experiments involving complex chromatographic conditions (7-9). For these reasons, along with the programming flexibility provided, we decided to use numerical integration to calculate solute retention times.

The program developed determines the useful gradient profile by approaching the chromatographic experiment in a stepwise fashion as the separation develops. The program flow chart is given in Figure 2-1A and 2-1B.

Knowledge of each solute's position in the column, the position of the gradient front and the distance which must be travelled prior to eluting from the column are essential pieces of information for determining which (if any) gradient profile will produce a desired chromatogram. The program begins by establishing which solute has travelled the greatest distance before being overtaken by the gradient. This solute is most likely to elute first and, therefore, the program will concentrate on determining the conditions required for eluting this solute. Using the capacity factor vs. mobile phase composition information, the possible solute band velocities can be established. Knowing the column length which must be travelled for this
Figure 2-1A. Program Flow Chart.

Start

Input isocratic retention data, gradient delay time, column length and flow rate.

Choose the initial percentage of the high strength solvent reservoir (%B).

Calculate the distance travelled by each solute during the gradient delay.

Calculate the distance which must be travelled by the lead solute before eluting.

Calculate the maximum and minimum velocity for the lead solute based on the isocratic retention data previously entered.

Using the distance the lead solute must travel and the limiting velocities, calculate the shortest and longest elution times for the solute if either an isocratic segment or a ramp segment is employed.

Inform the user of the elution limits for each segment type for the next solute to elute from the column.

Go to c on the next page.
Based on the user's choice of elution segment type and desired retention time, determine the actual elution conditions required.

Calculate the position of each solute in the column after the chosen elution profile passes it.

Has any solute overtaken the lead solute?

Yes

Inform the user and then repeat for the other solutes.

No

After all solutes have eluted display the final chromatogram. Provide the user with the gradient conditions which will produce the desired chromatogram.

Results satisfactory?

No

End

Yes

Both a and b are on the previous page.
solute to elute allows the program to establish the retention time limits for the solute if either a ramp or an isocratic step is employed.

Calculation and reporting of the retention time for the solute allows the user to determine what retention times are possible. The retention time limits for each solute can be calculated using the maximum and minimum capacity factors which are possible for the solute. The time limits for elution if an isocratic step is employed can be calculated using the velocity of the solute under these limiting conditions and the distance which must be travelled by the solute before eluting. It is assumed for these calculations that the isocratic segment is produced by an instantaneous change in mobile phase from one gradient segment to the next. This process is presented schematically in Figure 2-2.

Establishing the elution time limits for elution conditions where the mobile phase is linearly ramped from an initial solvent strength to a final solvent strength is more challenging. To determine the minimum retention time we chose to fix the starting conditions, and set the final concentration at the percent B which results in the largest $k'$ (i.e., the slowest velocity for the solute). The computer then increments the gradient slope and searches until the lead solute just elutes as the ramp segment is
Figure 2-2.
Schematic diagram indicating the approach taken to
determine the time range for elution using an
isocratic step. The solvent change for the step
ranges from the lowest to the highest strength mobile
phases to provide the retention limits of the solute.
ending. The same procedure is used to find the ramp segment which produces the maximum retention time for the solute, except the final percent B that is chosen is the one which results in the smallest k' for the solute (i.e., the fastest velocity for the solute). If the user requests that the solute elute with a retention time which is between the two limits, the program calculates the length of time required for the gradient segment and determines the final %B for the segment by searching between the %B's which produce the limiting k' values. This process is presented schematically in Figure 2-3.

Once the program establishes the retention time limits for both segment types, the user is informed of the possibilities. The user then directs the program to use either a ramp or isocratic segment and chooses a desired retention time for the solute within the appropriate time limits. Using the information provided by the user, the program calculates time duration for the gradient segment and determines the final %B for the gradient by searching between the %B's which produce the limiting k' values. The program then calculates the distances travelled by the remaining solutes as that segment of the gradient profile passes through the column.

One complicating factor is that during a gradient elution experiment the gradient front interacts with the
Figure 2-3.
Schematic diagram indicating the approach taken to determine the time range for elution using a ramp step. To determine the retention limits for the solute, the mobile phase ramp starts at the final %B of the previous segment. The final concentration of the ramp is allowed to range from the lowest to the highest strength mobile phase.
trailing solutes before reaching the lead solute. It is possible that one of these trailing solutes will overtake the "lead" solute and co-elute or pass the "lead" solute and elute before it. The program monitors for this possibility and informs the user if such a situation develops.

Once the gradient profile required to elute the "lead" solute at the time chosen is found, the process is repeated and the program calculates the elution time range for the next "lead" solute. This procedure is repeated until a multi-segment gradient profile, which elutes all of the solutes, is determined. The final step is to inform the user of the gradient profile which will produce the elution profile interactively determined.

2.3 Experimental

A. Instrumentation

The chromatograph consisted of a Nicolet LC/9560 low pressure mixing ternary gradient pumping system with a Nicolet LC/9563 variable wavelength UV/VIS detector (Nicolet Analytical Instruments, Madison, WI). Samples were injected using a Rheodyne Model 7125 injector having a 10 microliter sample loop (Rheodyne, Inc., Cotati, CA). A Kipp & Zonen B-D 40 series stripchart recorder (Kipp and Zonen, Holland) was used to record the analog output of the detector. A
Hewlett-Packard 3392A integrator (Hewlett-Packard, Avondale, PA) was also used to record the chromatograms and provided the retention times for the solutes. The HPLC column used for reversed phase HPLC study was packed with an octyl bonded phase (25 cm long, 5 micrometer particle size, 4.6 mm i.d.) (Supelco, Inc., Bellefonte, PA). A PRP-1 reversed phase column (150 mm x 4.1 mm) with a 5-10 μm particle size (macroporous polystyrene-divinylbenzene copolymers) (Hamilton Co., Reno NE) was used for pH gradient elution. The detector was set at 254 nm and the flow rate was set at 1.0 ml/min for this study. The gradient delay of the instrument was measured and found to be 3.6 minutes at this flow rate. The retention times presented for the gradient elution experiments are the average of two chromatographic runs.

All the calculations were performed using an AT&T 6300 microcomputer (AT&T, Bedminster, NJ) consisting of an 8 Mhz Intel 8086686 CPU, 640 kilobytes of RAM, and a 20 megabyte Winchester hard disk drive. The program was written in mostly in FORTRAN though the graphics routines were written in PASCAL.

B. Reagents

In reversed phase HPLC experiments, the mobile phases were prepared using HPLC-grade methanol and HPLC grade water
All mobile phases were filtered through a Nylon 66 membrane filter (with a pore size of 0.45 micrometer) and sparged with helium prior to use. The six test solutes used for reversed phase HPLC study were: 2,4-dinitrotoluene (U.S. Army Cold Regions Research and Engineering Laboratory Reference Standard, Hanover, NH), benzene (ACS Spectranalyzed, Fisher Scientific Company, Springfield, NJ), 1-bromo-4-nitrobenzene (Practical grade, Eastman Kodak, Rochester, NY), toluene (Reagent grade, Fisher Scientific, Springfield, NJ), chlorobenzene (Reagent grade, Eastman Kodak, Rochester, NY), diethylphthalate (Bakergrade, J.T. Baker, Phillipsburg, NJ).

In pH gradient elution experiments, citric acid and dibasic potassium phosphate (Fisher Scientific Co., Fair Lawn, NJ) were used to prepare the citric/phosphate buffer mobile phase. Both are anhydrous and reagent grade. The water used was HPLC grade. The mobile phase contained 0.01 M reagent grade potassium chloride (Fisher Scientific Co.) to control the ionic strength of the mobile phase. The pH of mobile phase reservoirs A and B were 4.64 and 7.69 respectively.

All solvents were filtered through a nylon filter membrane with pore size of 0.45 μm, then degassed using an ultrasonic bath and by sparging with helium for 10 minutes prior to use.
The test solutes were: 4-hydroxybenzoic acid (4-HBA), benzoic acid (BA) (Aldrich Chemical Company, Milwaukee, WI), 4-hydroxy-3-methoxybenzoic acid or vanillic (VAN), 3,4-dihydroxycinnamic acid or caffeic (CAFF), (Nutritional Biochemical Corp., Cleveland, OH) and 4-hydroxy-3,5-dimethoxybenzoic acid or syringic (SYG) (Sigma Co., St. Louis, MO). All solutes were reagent grade. The concentration of all samples was approximately 10 mg to 50 ml of HPLC grade water.

2.4 Results and Discussion

2.4.1 Reversed Phase HPLC

A series of experiments was performed to test the accuracy and applicability of this approach for determining suitable gradient elution conditions. The velocity of a solute can be calculated from the relationship between its capacity factor and the composition of the mobile phase. To obtain this relationship, isocratic retention data were acquired for each of the six test solutes at seven isocratic mobile phase conditions, ranging from 72% methanol (80% pump B) to 41% methanol (20% pump B). These isocratic retention data are given in Table 2-1.

The test solutes were chosen due to the complexity of their capacity factor relationships. A number of solutes
<table>
<thead>
<tr>
<th>Solute</th>
<th>20%</th>
<th>30%</th>
<th>40%</th>
<th>50%</th>
<th>60%</th>
<th>70%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-Dinitrotoluene</td>
<td>22.95</td>
<td>15.83</td>
<td>11.41</td>
<td>8.41</td>
<td>6.52</td>
<td>5.25</td>
<td>4.40</td>
</tr>
<tr>
<td>Benzene</td>
<td>21.20</td>
<td>15.83</td>
<td>11.77</td>
<td>9.00</td>
<td>7.11</td>
<td>5.78</td>
<td>4.85</td>
</tr>
<tr>
<td>Bromobenzene</td>
<td>37.46</td>
<td>24.84</td>
<td>16.60</td>
<td>11.58</td>
<td>8.45</td>
<td>6.44</td>
<td>5.14</td>
</tr>
<tr>
<td>Toluene</td>
<td>45.06</td>
<td>30.77</td>
<td>20.86</td>
<td>14.40</td>
<td>10.40</td>
<td>7.76</td>
<td>6.04</td>
</tr>
<tr>
<td>Chlorobenzene</td>
<td>48.50</td>
<td>32.30</td>
<td>20.86</td>
<td>14.40</td>
<td>10.21</td>
<td>7.50</td>
<td>5.82</td>
</tr>
<tr>
<td>Diethylphthalate</td>
<td>59.50</td>
<td>32.40</td>
<td>18.82</td>
<td>11.60</td>
<td>8.12</td>
<td>5.99</td>
<td>4.84</td>
</tr>
</tbody>
</table>

Table 2-1. Isocratic data for the six test solutes.
having overlapping or crossing capacity factor vs. mobile phase composition relationships were chosen for this study. The reason for choosing such test solutes was that if the plots of capacity factor vs. mobile phase composition for two solutes crossed, then the order of elution for the solutes was dependent on the mobile phase composition of the experiment. Including such complex relationships provided a more challenging test of the capabilities of the program and approach. Since the goal in developing this approach was to provide the user with increased control in producing a desired separation, a number of desired separations were evaluated.

The following examples demonstrate how a scientist could use this approach for methods development to determine the elution conditions which produce the chromatographic results desired. It should be noted that the ability to produce separation depends on the capacity factor vs. mobile phase relationships of the solutes. Therefore, the scientist must keep in mind that not every desired separation is chromatographically viable no matter which approach is used for methods development.

The goal of the first set of simulated experiments was to determine a multi-segment elution profile capable of producing a separation of each of the six test solutes. The initial mobile phase composition was set at 20% B for the
experiment. Using the initial mobile phase concentration, the isocratic retention data given in Table 2-1 and the instrument delay volume, the program determined that benzene would travel the farthest in the column during the delay volume. Since benzene was determined to be the "lead" solute, the next step was for the program to calculate the retention time limits for eluting this solute with either an isocratic segment or a ramp segment. The program performed the calculations and informed the user that the retention time limits for benzene were from 7.6 to 21.2 minutes if an isocratic step were chosen, and 10.3 to 21.2 minutes if a gradient ramp were chosen. At this point, the user chose to elute the solute with a ramp segment and with a retention time of 19.5 minutes. The program calculated the actual ramp segment required to elute benzene at 19.5 minutes and using this information determined the position of each remaining solute in the column after the segment used to elute the benzene passed it. These calculations also determined that no other solute in the column had overtaken benzene during this gradient segment.

At this point, the program began to calculate the retention time limits for the new "lead" solute, 2,4-dinitrotoluene, since it had travelled the largest distance in the column and was, therefore, the most likely to elute next. The program informed the user that the retention time
limits for 2,4-dinitrotoluene would range from 19.6 to 20.6 minutes if the next segment were of the isocratic type or from 19.8 to 20.5 minutes if the next segment were a ramp. The user elected to elute 2,4-dinitrotoluene with a ramp segment and chose a retention time of 20.4 minutes for the compound.

Continuing in an interactive mode, the program determined the ramp segment needed to elute 2,4-dinitrotoluene at 20.4 minutes, again calculated the positions of each of the solutes which remained in the column. The program informed the user that 1-bromo-4-nitrobenzene would be the third solute to elute. The interactive session proceeded as above with the user choosing to alternate between ramp and isocratic segments for this particular multi-segment elution profile. The retention times chosen for solutes number three through six were: 31.8, 37.7, 39.9 and 42.8 minutes, respectively. It is important to note that the order of elution for these compounds, given from earliest to latest eluting was: benzene; 2,4-dinitrotoluene; 1-bromo-4-nitrobenzene; toluene, chlorobenzene and diethylphthalate. A summary of this interactive session including the desired and experimentally obtained retention times is presented in Table 2-2. The gradient profile as determined by the program for producing the desired chromatogram is given in
<table>
<thead>
<tr>
<th>Order of Element</th>
<th>Solvent Name</th>
<th>Retention Time Range (minutes)</th>
<th>Isocratic Segment</th>
<th>Retention Time Range (minutes)</th>
<th>Ramp Segment</th>
<th>Segment Desired (I = Isocratic Step, R = Ramp Step)</th>
<th>Desired Retention Time (minutes)</th>
<th>Actual Retention Time (minutes)</th>
<th>% Difference Between Actual and Desired Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Benzene</td>
<td>7.6-21.2</td>
<td>16.3-21.2</td>
<td>R</td>
<td>19.5</td>
<td>19.3</td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>2.</td>
<td>2,4- Dimethylaniline</td>
<td>19.6-20.6</td>
<td>19.8-20.5</td>
<td>R</td>
<td>20.4</td>
<td>20.1</td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>3.</td>
<td>1-Bromo-4-Nitro Benzene</td>
<td>21.7-34.8</td>
<td>23.6-34.9</td>
<td>R</td>
<td>31.8</td>
<td>30.9</td>
<td></td>
<td></td>
<td>7.8</td>
</tr>
<tr>
<td>4.</td>
<td>Toluene</td>
<td>32.6-39.7</td>
<td>33.5-39.9</td>
<td>R</td>
<td>37.7</td>
<td>36.7</td>
<td></td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td>5.</td>
<td>Chlorobenzene</td>
<td>38.0-48.5</td>
<td>38.5-48.1</td>
<td>R</td>
<td>39.9</td>
<td>39.7</td>
<td></td>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td>6.</td>
<td>Dicyclohexane</td>
<td>48.3-49.3</td>
<td>48.8-49.6</td>
<td>R</td>
<td>42.8</td>
<td>42.4</td>
<td></td>
<td></td>
<td>0.9</td>
</tr>
</tbody>
</table>

Table 2-2.
Predicted retention time ranges for isocratic and ramp segments in Example 1. The retention times chosen by the user for each solute and the experimental retention times are provided for comparison.
A second example demonstrates the control which the user can have when developing a separation using this interactive approach. After arriving at the conditions which produced the separation described in Table 2-2, we decided to try to arrive at elution conditions which would result in a separation of the six test solutes in less time. The initial solvent strength was increased to 60% B for these experiments.

As in the first example, the program began by calculating the distance travelled by each solute during the delay time. It determined that the first compound to elute for these initial conditions would not be benzene, as in the previous example, but instead was most likely to be 2,4-dinitrotoluene since it had travelled the farthest in the column during the delay time. The program next calculated that the retention time limits for 2,4-dinitrotoluene ranged from 6.1 to 10.1 minutes if eluted with an isocratic step and from 6.3 to 7.5 minutes if eluted with a ramp segment. The user directed the program to have 2,4-dinitrotoluene elute using a ramp segment with a retention time of 6.5 minutes.

The program determined the ramp segment required to produce this elution time for the compound. It then
Figure 2-4.
Gradient profile as determined by the program for producing the separation described in Table 2-2.
Figure 2-5. Simulated chromatogram produced using the gradient profile given in Figure 2-4.

a = benzene
b = 2,4-dinitrotoluene
c = 1-bromo-4-nitrobenzene
d = toluene
e = chlorobenzene
f = diethylphthalate
determined the positions of the other solutes in the column once that particular ramp segment had passed and found that benzene was the new "lead" solute and was therefore expected to be the second solute to elute. The program determined that the retention times possible for benzene ranged from 6.8 to 8.7 minutes or from 7.0 to 7.5 minutes if an isocratic or ramp segment were chosen, respectively. The user in this case decided to use a ramp segment and to have benzene elute at 7.2 minutes. The process continued, with the user choosing to employ isocratic segments to elute the last four solutes. A summary of this process is presented in Table 2-3. The gradient profile determined and the resulting chromatogram are given in Figures 2-6 and 2-7, respectively.

A very interesting feature demonstrated by these two experiments should be noted. For these test solutes, manipulating the elution profile allowed the user to change the order of elution of the compounds. The elution order for the chromatogram presented in Figure 2-7 was quite different from that obtained in the previous example.

The goal of most chromatographers during methods development is to arrive at elution conditions which completely separate the solutes in a given sample. There are times, however, when it is reasonable to allow some of the solutes, such as those for which information is not
<table>
<thead>
<tr>
<th>Order of</th>
<th>Solute Name</th>
<th>Retention Time Range (minutes) Isocratic Segment</th>
<th>Retention Time Range (minutes) Ramp Segment</th>
<th>Segment Desired (R = Ramp Step) Desired Retention Time (minutes)</th>
<th>Actual Retention Time (minutes)</th>
<th>% Difference Between Actual and Desired Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,4-Dimethyl Toluene</td>
<td>6.1-10.1</td>
<td>6.3-7.3</td>
<td>R</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>2</td>
<td>Benzene</td>
<td>6.8-8.7</td>
<td>7.0-7.5</td>
<td>R</td>
<td>7.2</td>
<td>7.0</td>
</tr>
<tr>
<td>3</td>
<td>Decylphthalate</td>
<td>7.7-17.0</td>
<td>8.1-11.4</td>
<td>I</td>
<td>7.9</td>
<td>7.9</td>
</tr>
<tr>
<td>4</td>
<td>1-Bromo-4-Nitro Benzene</td>
<td>8.1-9.8</td>
<td>8.2-8.3</td>
<td>I</td>
<td>8.6</td>
<td>8.2</td>
</tr>
<tr>
<td>5</td>
<td>Chlorobenzene</td>
<td>9.6-10.3</td>
<td>10.2-10.1</td>
<td>I</td>
<td>9.5</td>
<td>9.7</td>
</tr>
<tr>
<td>6</td>
<td>Toluene</td>
<td>9.6-10.6</td>
<td>9.6-9.8</td>
<td>I</td>
<td>10.2</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Table 2-3.

Predicted retention time ranges for isocratic and ramp segments in Example 2. The retention times chosen by the user for each solute and the experimental retention times are provided for comparison.
Figure 2-6.
The Gradient profile as determined by the program for producing the separation described in Table 2-3.
Figure 2-7. Actual chromatogram produced using the gradient profile given in Figure 2-6.

- a = 2,4-dinitrotoluene
- b = benzene
- c = diethylphthalate
- d = 1-bromo-4-nitrobenzene
- e = chlorobenzene
- f = toluene
required, to co-elute. Determining elution conditions which force two or more solutes to co-elute might be one way to reduce the time required for a chromatographic run. Arriving at such conditions might also be useful for analyses where the goal is to determine a value for the total concentration of two or more solutes rather than their individual concentrations.

To demonstrate the control which this approach provides the user in arriving at conditions which produce separations involving co-elution of selected components, it was decided that benzene and 2,4-dinitrotoluene, the earliest eluting solutes did not need to elute separately for this analysis. Therefore, the user attempted to determine elution conditions which forced these two solutes to co-elute while still allowing the remaining solutes to be separated. The user informed the program to start with an initial mobile phase of 30% B. The program responded that the first solute to elute would be benzene. The program then calculated that the retention time range for benzene is between 7.4 and 19.8 minutes using an isocratic segment or between 9.3 and 17.7 minutes if the solute is eluted using a ramp segment. The user decided to have benzene elute at 16.0 minutes with a ramp segment. The program determined that using this ramp segment would result in 2,4-dinitrotoluene co-eluting with benzene which was one of the goals of this experiment.
The simulation process continued in an effort to find elution conditions which separated the remaining solutes. The program determined that the third solute would elute between 17.3 and 29.7 minutes if the next segment were an isocratic step, or between 18.8 and 27.1 minutes if a ramp segment were employed. The user decided to elute the third solute at 28.0 minutes with an isocratic segment. Eventually, an elution profile which separated each of the remaining bands was established. The final elution profile is given in Figure 2-8. The simulated and actual chromatograms are presented in Figure 2-9.

The elution conditions which produce a given separation are not necessarily unique. Once conditions which produce a desired chromatographic result are established in the laboratory, however, it is usually difficult to justify the additional investment of time required to attempt to determine other elution profiles that produce the same or similar results. There are times, however, when the scientist may prefer to employ one elution profile instead of another. In such cases, chromatographic simulation using the approach described here may prove to be of significant advantage. For example, an elution profile was developed consisting of all isocratic segments which separated the first two solutes while producing one band for the third and fourth solutes and one band for the fifth and
Figure 2-8.
Gradient profile as determined by the program for producing the separation given in Example 3.
Figure 2-9.
The simulated (top) and experimentally obtained (bottom) chromatograms for Example 3.
sixth solutes. The elution profile and resulting chromatogram are given in Figures 2-10 and 2-11, respectively. The first compound to elute under these conditions is 2,4-dinitrotoluene and the second compound to elute is benzene. Two compounds, 1-bromo-4-nitrobenzene and diethylphthalate, co-elute to give the third band, while toluene and chlorobenzene co-elute to give the fourth band.

Chromatographic simulation, using the approach described, provides the scientist with a reasonably easy method for determining if other elution conditions can produce similar chromatographic results. In this case, it was decided to determine if the compounds could be eluted in the same retention order and times using a gradient profile consisting of multiple ramp segments instead of multiple isocratic segments. Using the program, it was determined that the multi-ramp elution profile presented in Figure 2-12 would produce the desired chromatographic results. The actual chromatographic results confirmed the simulated experiments.

2.4.2 pH Gradient Elution

The approach to multi-segment gradient elution methods development was also tested under pH gradient elution conditions. Table 2-4 gives the percentages of pump B and the pHs of the mobile phase vs the retention times of the
Figure 2-10.
Gradient profile as determined by the program for producing the separation given in Example 4.
Figure 2-11.

Experimentally determined chromatograph produced using the elution conditions presented in Figure 2-10.
Figure 2-12.
Gradient profile, containing all ramp segments, which the program calculated would provide the same solute retention times as the profile given in Figure 2-10.
### Table 3-4. Isocratic data for pH gradient elution.

<table>
<thead>
<tr>
<th>Percent B</th>
<th>20%</th>
<th>25%</th>
<th>30%</th>
<th>40%</th>
<th>50%</th>
<th>60%</th>
<th>70%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.36</td>
<td>5.63</td>
<td>5.82</td>
<td>6.19</td>
<td>6.49</td>
<td>6.74</td>
<td>6.94</td>
<td>7.14</td>
</tr>
<tr>
<td>Test solute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-HBA</td>
<td>9.35</td>
<td>6.28</td>
<td>4.45</td>
<td>2.87</td>
<td>2.27</td>
<td>2.01</td>
<td>1.91</td>
<td>1.82</td>
</tr>
<tr>
<td>VAN</td>
<td>42.30</td>
<td>24.77</td>
<td>16.69</td>
<td>8.18</td>
<td>5.56</td>
<td>4.59</td>
<td>3.82</td>
<td>3.43</td>
</tr>
<tr>
<td>CAFF</td>
<td>30.21</td>
<td>18.06</td>
<td>13.81</td>
<td>7.61</td>
<td>5.56</td>
<td>4.60</td>
<td>4.21</td>
<td>3.92</td>
</tr>
<tr>
<td>STG</td>
<td>57.02</td>
<td>34.07</td>
<td>23.66</td>
<td>11.83</td>
<td>8.22</td>
<td>6.56</td>
<td>5.22</td>
<td>4.56</td>
</tr>
<tr>
<td>MA</td>
<td>81.96</td>
<td>48.24</td>
<td>33.30</td>
<td>17.10</td>
<td>11.81</td>
<td>9.30</td>
<td>6.03</td>
<td>7.23</td>
</tr>
</tbody>
</table>
five solutes for isocratic elution conditions.

The initial conditions were set at 20% pump B for all experiments. The retention times for the five solutes were set at 7.5, 13.5, 14.7, 17.0 and 19.4 minutes for both runs. Figures 2-13 and 2-14 show the elution profiles produced by the program and used for these two experiments. All isocratic segments were used for the first run and all ramp segments were used for the second run. The first two separation results are presented in Tables 2-5 and 2-6. From Tables 2-5 and 2-6 it is clear that there was good agreement between the actual and the desired results.

The results of three additional experiments are presented in Table 2-7, where the desired and the actual retention times for the five solutes were very close. In these three examples, the desired retention times for the five solutes were the same and the obtained chromatograms were very similar. In one of the experiments, all ramp segments were used. In another experiment both isocratic and ramp segments were used, and in the third example all isocratic segments were used. Figure 2-15 shows the elution profiles and the chromatograms for the three runs. The desired retention times for 3,4-dihydroxycinnamic acid and 4-hydroxy-3-methoxybenzoic acid were 9.3 and 9.5 minutes, and the two solutes were co-eluted.

These examples indicated the accuracy of the approach.
Figure 2-13. pH Gradient profile containing all the isocratic segments needed to produce the separation described in Table 2-5.
Figure 2-14. pH Gradient profile containing all the ramp segments needed to produce the separation described in Table 2-6.
Desired and Actual Retention Times (min) Under pH Gradient Elution Conditions

<table>
<thead>
<tr>
<th>Solute</th>
<th>Desired</th>
<th>Actual</th>
<th>Error(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-HBA</td>
<td>7.5</td>
<td>7.5</td>
<td>---</td>
</tr>
<tr>
<td>CAFF</td>
<td>13.5</td>
<td>13.4</td>
<td>0.7</td>
</tr>
<tr>
<td>VAN</td>
<td>14.7</td>
<td>14.7</td>
<td>---</td>
</tr>
<tr>
<td>SYG</td>
<td>17.0</td>
<td>17.1</td>
<td>0.6</td>
</tr>
<tr>
<td>BA</td>
<td>19.4</td>
<td>19.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 2-5.
Separation results using pH gradient elution conditions containing all isocratic segments presented in Figure 2-13.
Desired and Actual Retention Times (min) Under pH Gradient Elution Conditions

<table>
<thead>
<tr>
<th>Solute</th>
<th>Desired</th>
<th>Actual</th>
<th>Error(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-HBA</td>
<td>7.5</td>
<td>7.5</td>
<td>---</td>
</tr>
<tr>
<td>CAFF</td>
<td>13.0</td>
<td>13.1</td>
<td>0.8</td>
</tr>
<tr>
<td>VAN</td>
<td>14.0</td>
<td>14.1</td>
<td>0.7</td>
</tr>
<tr>
<td>SYG</td>
<td>16.5</td>
<td>16.7</td>
<td>1.2</td>
</tr>
<tr>
<td>BA</td>
<td>19.0</td>
<td>19.1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Table 2-6.
Separation results using pH gradient elution conditions containing all ramp segments presented in Figure 2-14.
Desired and Actual Retention Times (min) Under pH Gradient Elution Conditions

<table>
<thead>
<tr>
<th>Solute</th>
<th>Desired</th>
<th>Actual</th>
<th>Error(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-HBA</td>
<td>6.5</td>
<td>1) 6.4</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 6.5</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3) 6.5</td>
<td>0.3</td>
</tr>
<tr>
<td>CAFF</td>
<td>9.3</td>
<td>1) 9.3</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 9.5</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3) 9.5</td>
<td>2.3</td>
</tr>
<tr>
<td>VAN</td>
<td>9.5</td>
<td>1) 9.3</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 9.5</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3) 9.5</td>
<td>0.2</td>
</tr>
<tr>
<td>SYG</td>
<td>11.3</td>
<td>1) 11.3</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 11.5</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3) 11.5</td>
<td>1.7</td>
</tr>
<tr>
<td>BA</td>
<td>14.3</td>
<td>1) 14.2</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 14.5</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3) 14.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Table 2-7.
Separation results using pH gradient elution conditions containing 1) all ramp segments, 2) both ramp and isocratic segments, 3) all isocratic segments.
pH Gradient profiles, containing all ramp segments (top); both ramp and isocratic segments (middle); all isocratic segments (bottom), and experimentally obtained chromatograms.
for these experiments using pH gradient elution condition. The mobile phases for these experiments contained no organic modifier. The separations were controlled by changing only the pH of the mobile phase.

2.5 Summary

The accuracy and versatility of an approach for interactively determining multi-segment gradient elution profiles has been demonstrated. The key feature of this approach to chromatographic methods development is the ability to inform the user of reasonable retention limits for each solute and to determine an elution profile capable of eluting each solute within a specified set of retention limits. The scientist using this technique for HPLC methods development can approach the problem in a fundamentally different way than that which is normally practiced in the laboratory. Work continues to: 1.) incorporate more complex elution segments, 2.) develop post-simulation algorithms which allow the user to reduce the complexity of the elution profile and 3.) develop the knowledge necessary to incorporate the approach developed into a knowledge-based system for HPLC methods development.
CHAPTER 3

COMPUTER-ASSISTED METHODS DEVELOPMENT FOR INCREASING DETECTION SENSITIVITY FOR MULTI-SEGMENT GRADIENT ELUTION HPLC EXPERIMENTS

3.1 Introduction

Detectability is especially important for trace analysis using HPLC. The magnitude of the response of the detector for a given solute depends on the concentration or mass of the solute entering the detector per unit of time. It should be noted that while the peak height and width change with the elution conditions, the product of the two parameters is almost constant (108). In general, the higher the peak height, the greater the detectability of the solute will be. It is very hard to detect a trace solute if its peak height is very small. Its peak might be lost in the baseline. In general, to improve detection sensitivity, the peak width should be made as narrow as possible, so that the peak height is as large as possible.

The width of a chromatographic peak in an elution process is a function of two factors, the physical width of the solute zone on the column as it elutes and the speed at which it elutes (98). The width of the solute zone on the
The column efficiency or number of theoretical plates produced by the column is directly related to the number of theoretical plates produced by the column (99). The velocity of the solute zone as it elutes from the column depends on the capacity factor, \( k' \), at that point. A smaller capacity factor will result in the solute eluting with a narrower band width. The smaller the \( k' \), the narrower the peak width will be. The \( k' \) value of the zone depends on the strength of the mobile phase. Thus, using a higher strength mobile phase results in a smaller \( k' \) for the solute and an increased peak height.

The ability of a column to resolve two solutes is of prime interest in HPLC. The selectivity factor, \( \alpha \), for two solutes is defined as

\[
\alpha = \frac{K_2}{K_1}
\]

(3-1)

where \( K_2 \) is the partition coefficient of the more strongly retained solute 2 and \( K_1 \) is the partition coefficient of the less strongly held and therefore more rapidly moving solute 1. By definition, \( \alpha \) must always be greater than or equal to one. The capacity factor is defined as:

\[
k' = \frac{K_2 V_s}{V_M}
\]

(3-2)

where \( V_s \) and \( V_M \) are the total volumes of the stationary phase.

79
and the mobile phase in the column, respectively. This equation rearranges to

\[ K_1 = \frac{k'_1 \times V_M}{V_s} \quad (3-3) \]

and

\[ K_2 = \frac{k'_2 \times V_M}{V_s} \quad (3-4) \]

Substitution of these two Equations into 3-1 provides a relationship between the selectivity factor and the capacity factor. That is,

\[ \alpha = \frac{k'_2}{k'_1} \quad (3-5) \]

Usually, the difference in the \( k' \) values of two solutes decreases if they are eluted using a high strength mobile phase. As a result, the two solutes will have a smaller \( \alpha \) when a greater strength mobile phase is used to separate the two compounds. A typical example is given in Figure 3-1, which shows the chromatograms of five solutes eluted by a stronger and a weaker strength mobile phase, respectively. Figure 3-1A gives the chromatogram obtained for an isocratic experiment using an aqueous mobile phase.
Figure 3-1.

Chromatograms obtained by isocratic elution. Methanol concentrations were 64% in 3-1A (top) and 31% in 3-1B (bottom).
that contains 64% methanol. Figure 3-1B gives the chromatogram obtained when using a lower strength mobile phase, 31% methanol, to isocratically elute the solutes. The peaks in Figure 3-1A were clearly sharper and have a narrower peak width when eluted using the stronger mobile phase. Some of the solutes overlapped (or coeluted) under these elution conditions. It should also be noted that smaller α's were obtained. It is clear from Figure 3-1B that the lower strength mobile phase produced greater differences in retention times but the peak widths were clearly much broader.

Alternately, one could consider using a lower strength mobile phase during the early elution process to obtain sufficient separation of the solutes and using the highest strength mobile phase for a short period of time when the solute is close to the end of the column, to elute it. The solute zone will then be accelerated to a higher velocity and eluted into the flow cell in a more concentrated band. Peak height will increase as the concentration in the flow cell increases if the chromatograph employs a concentration dependent detector. This is more important for the later eluting compounds. The approach to increasing detection sensitivity is often called the "zone compression effect" (98, 170). For zone compression to be achieved, the solute must be close to the end of the column when the high
strength mobile phase, which will be used for elution, reaches it.

Using this approach, detection sensitivity can be maximized while producing a satisfactory separation. Potential applications where zone compression may prove to be important include preparative chromatography and in combined liquid chromatography / mass spectrometry (LC/MS) experiments. The approach can be used in preparative chromatography to increase collection efficiency. In LC/MS, when using "zone compression" a more concentrated solute band will enter the mass spectrometer, thereby improving the signal to noise ratio for the mass spectrum obtained.

Previous work concentrated on developing an approach for computer-assisted methods development for multi-segment gradient elution experiments. Based on the results obtained in that work, it was decided to extend the approach in order to provide the user with the option of using zone compression to improve the detection sensitivity for solutes being separated. The programs developed can be used not only to tell the user the gradient profiles for desired retention times, but also to provide gradient profiles which meet the user's "zone compression" requirements for some solutes while keeping the retention times for the rest of the solutes unchanged.
Theory

Several assumptions must be made to develop an approach which utilizes zone compression to improve detection sensitivity. These assumptions are as follows:

1. The theoretical plate number is approximately constant for each solute in a mixture for a given set of experimental conditions (a particular column and mobile phase, mobile phase velocity and temperature).
2. The theoretical plates obtained for isocratic elution experiments are generally considered to be independent of the capacity factors of the solutes (99).
3. Solute bands eluting from a column are Gaussian-shaped.
4. The responses of the detector are in the linear range for the experimental conditions.

Once these assumptions have been made, a number of simple calculations can be used to determine the on-column band width of the solutes just prior to their eluting from the column. If \( 4\sigma \) is the width of the sample bands just before it leaves the column, where \( \sigma \) is the standard deviation of the Gaussian distribution in units of length (cm), then the plate height \( H \) can be calculated by (116):
where $L$ is the length of the column in cm and $N$ is the plate number.

The value of $N$ is generally assumed to be independent of the capacity factor $k'$. Thus

$$\sigma = \frac{L}{\sqrt{N}} = \text{constant} \quad (3-8)$$

The widths of the sample bands, $4\sigma$, are a constant for different solutes and for different mobile phases of similar viscosity. In other words, to a first approximation different solutes reach the end of the column before being eluted with approximately the same physical zone width (98, 99, 23).

Detection of a Gaussian-shaped solute band eluting from a column will depend on the ability to sense the concentration at the peak maximum of the solute band as it elutes from the column. The concentration at peak maximum of the solute, $C_{\text{max}}$, can be calculated using the equation

$$H = \frac{L}{N} = \frac{\sigma^2}{L} \quad (3-6)$$

So,

$$N = \left( \frac{L}{\sigma} \right)^2 \quad (3-7)$$
where $m$ is the mass, in mole, of the solute injected into the column, $N$ is the number of theoretical plates of the column and $V_R$ is the retention volume, in ml, of a solute band as it elutes from the column.

$V_R$ can be obtained using:

$$V_R = F \times t_R$$  \hspace{2cm} (3-2)

where $F$ is the volumetric flow rate of the mobile phase in ml min$^{-1}$; and $t_R$ is the retention time, in min, of the solute.

From the fundamental equation the retention time of a solute can be calculated using:

$$t_R = t_M (1 + k')$$  \hspace{2cm} (3-3)

Combining equations (3-1), (3-2) and (3-3) then yield:

$$C_{\max} = \frac{m/\sqrt{N}}{V_R/2\pi}$$  \hspace{2cm} (3-4)

The most common type of detector used in HPLC is a UV absorption detector. The absorption signal measured by such detectors is directly related to the concentration of the solute in the detector cell. The maximum concentration of
a solute, $C_{\text{max}}$, determines the peak height observed for that solute. According to the Beer-Lambert law, the absorbance measured by the UV detector can be calculated by:

$$A = \varepsilon C_{\text{max}} d$$  \hspace{1cm} (3-5)

where $C_{\text{max}}$ is the concentration at the peak maximum, in mol l$^{-1}$, d is the path length in cm, and $\varepsilon$ is the molar absorptivity in L cm$^{-1}$ mol$^{-1}$. By substituting equation (3-4) into equation (3-5) results in:

$$A = \frac{\varepsilon d m \sqrt{N}}{F c_m (1+k') \sqrt{2\pi}}$$  \hspace{1cm} (3-6)

It is clear that the detector signal is in inversely proportion to $(1 + k')$ and increases in approximate proportion to the solvent strength as the band exits the column. Thus, to a first approximation, peak height is dependent only on the strength of the mobile phase as the solute leaves the column, not on the mobile phase strength that the solute experiences while eluting through the column.

3.3 Program Description

The goal of this work was to develop an approach which allows the chromatographer to determine gradient elution profiles which result in user specified retention times for the solutes and also provide increased detection sensitivity for specific solutes, when necessary to meet the
requirements of the user. Detection sensitivity is increased by the zone compression effect, which results in a higher concentration of the solute reaching the detector cell.

The underlying principles of the general approach were described in Chapter 1. This approach to HPLC methods development is based on knowing the position of each solute in the column at any time. The program developed calculates the velocity, and corresponding capacity factor, which is required for the solute to reach the end of the column. The mobile phase concentration which produces the required capacity factor is then determined based on the isocratic elution data provided for the solutes. To compress the solute zone, and thereby increase the concentration of the solute prior to entering the detector cell, a 0.4 minute segment of the highest strength mobile phase for the solute is programmed to reach the solute just as it elutes from the column. Solutes to be zone compressed are chosen by the user.

A 0.4 minute high strength elution segment was used for these studies. This time length was determined empirically based on two opposing factors. First, if the segment of higher strength solvent is too long, it will cause other bands on the column to overlap. Second, if the duration time is too short, then the possibility of missing "zone
compression increases.

3.4 Experimental

A. Equipment

The chromatograph used for these studies was the one described in Chapter 2. The separations were performed at ambient temperature with a 25 cm x 4.6 mm column with 5 micron octyl-packing material (SUPELCO, INC., Supelco Park, Bellefonte, PA).

B. Chemicals

Mobile phases consisted of HPLC-grade methanol and water. Both were from Fisher Scientific (Springfield, NJ). A and B reservoirs contained 20 and 75 percent methanol, respectively.

A mixture of five compounds was used as the test solution. It consisted of: benzene, 1-bromo-4-nitrobenzene, toluene, chlorobenzene, and o-nitrotoluene (Reagent grade, Eastman Kodak, Rochester, NY).

Before use, all solvents were filtered through a 0.45 
\mu m membrane and sparged with helium for five minutes to degas.

3.5 Results and Discussion
Our ability to use the zone compression effect during the methods development process was examined using a series of multi-segment gradient elution HPLC experiments. The isocratic data obtained experimentally for the five test solutes used in this study are given in Table 3-1. The program developed used these data to calculate capacity factors from intermediate mobile phases by linear interpolation.

In the first experiment, the initial concentration of the mobile phase was set at 20% of reservoir B. It was decided to develop a separation of these solutes first without using "zone compression" for any of the solutes. The retention times chosen for the five solutes were 15.0, 21.0, 24.0, 26.0 and 28.0 minutes. All of these retention times were determined to be within the retention time ranges given by the program for each solute. It was further decided to use ramp segments for all five elution steps. The program based on this information determined the multi-segment gradient elution profile that provided the desired retention times for the test solutes. The elution profile is given in Figure 3-2. The actual retention times produced using these elution conditions for the five solutes were: 15.0, 20.8, 23.8, 25.9 and 27.9 minutes, respectively. The corresponding capacity factors for the solutes as they eluted from the column were: 3.4, 5.1, 7.0, 4.8 and 8.5.
<table>
<thead>
<tr>
<th>Test solute</th>
<th>Percent B</th>
<th>20%</th>
<th>30%</th>
<th>40%</th>
<th>50%</th>
<th>60%</th>
<th>70%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzene</td>
<td></td>
<td>16.73</td>
<td>13.75</td>
<td>11.14</td>
<td>9.04</td>
<td>7.40</td>
<td>6.22</td>
<td>5.37</td>
</tr>
<tr>
<td>o-nitro toluene</td>
<td></td>
<td>28.01</td>
<td>20.48</td>
<td>14.99</td>
<td>11.15</td>
<td>8.51</td>
<td>6.80</td>
<td>5.64</td>
</tr>
<tr>
<td>1-bromo-4-nitrobenzene</td>
<td></td>
<td>32.86</td>
<td>23.93</td>
<td>17.40</td>
<td>12.80</td>
<td>9.61</td>
<td>7.53</td>
<td>6.12</td>
</tr>
<tr>
<td>toluene</td>
<td></td>
<td>35.29</td>
<td>26.71</td>
<td>19.91</td>
<td>14.83</td>
<td>11.36</td>
<td>8.73</td>
<td>6.96</td>
</tr>
<tr>
<td>chlorobenzene</td>
<td></td>
<td>40.29</td>
<td>29.70</td>
<td>21.51</td>
<td>15.59</td>
<td>11.30</td>
<td>8.73</td>
<td>6.96</td>
</tr>
</tbody>
</table>

Table 3-1. Isocratic data used in the zone compression experiments.
Figure 3-2.
Gradient profile as determined by the program for producing the separation in the first experiment without using "zone compression".
The second experiment investigated conditions which would cause the second compound (o-nitrotoluene) to be compressed, while maintaining the retention times for the first two solutes at 15.0 and 21.0 minutes. The program reported that it would be impossible to keep the retention times completely unchanged for the rest of the solutes after the second solute had been compressed. It was, therefore, decided to determine an elution profile which would result in retention times for the later eluting solutes which were close to those obtained in the first experiment.

The user set the retention times for the other three solutes at 22.1, 24.0 and 26.0 minutes. Figure 3-3 gives the elution profile which was determined by the program. It shows that this elution profile incorporates a short segment of the highest strength mobile phase which is used to zone compress and elute the second peak. Using this elution profile resulted in the second solute band being compressed as it eluted providing a peak height which was 0.93 times higher than in the first experiment. The calculated capacity factor decreased from 5.1 in the first experiment to 0.99 in the second experiment. The actual retention times for the solutes in the second experiment were: 15.0, 20.2, 22.36, 24.16 and 26.69 minutes. The chromatograms for the first run, in which there was no "zone compression", and
Figure 3-3.
Gradient profile as determined by the program for producing the separation in the second experiment using "zone compression" for the second peak.
the second run, in which the second peak was compressed, are given in Figure 3-4. The desired and experimental retention times for this experiment are presented in Table 3-2.

As described previously, an estimate of the magnitude of the increase in absorption measured for a compressed solute band can be calculated using equation 3-6. If $A_1$ and $A_2$ are the UV detector signals for the first and second experiments, respectively, then the ratio of the absorption maxima for the two experiments can be determine by solving the following equation:

$$\frac{E_2}{E_1} = \frac{1+k_1}{1+k_2} \frac{1+5.1}{1+0.99} = 3$$

The solution for these experiments indicates that the second peak should have three times the absorbance of the peak in the first experiment. Experimentally, the absorbance for o-nitrotoluene in the second experiment was found to be only about twice that in the first experiment.

A third experiment was performed using zone compression for benzene, the solute which eluted first, and setting the retention times of the five solutes at 15.0, 21.0, 24.0, 26.0 and 28.0 minutes. The program determined the gradient profile which results in these retention times. The elution profile is given in Figure 3-5. Figure 3-6 presents the results from the chromatogram obtained using
Figure 3-4.

The chromatograms for the first run (top), in which there was no "zone compression", and the second run (bottom), in which the second peak was compressed.
Desired and Actual Retention Times (min) in the Zone Compression Experiments

<table>
<thead>
<tr>
<th>Solute</th>
<th>Desired</th>
<th>Actual</th>
<th>Error(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzene</td>
<td>15.0</td>
<td>15.0</td>
<td>---</td>
</tr>
<tr>
<td>o-nitro-toluene</td>
<td>21.0</td>
<td>20.2</td>
<td>3.9</td>
</tr>
<tr>
<td>1-bromo-4-nitrobenzene</td>
<td>22.1</td>
<td>22.4</td>
<td>1.2</td>
</tr>
<tr>
<td>toluene</td>
<td>24.0</td>
<td>24.2</td>
<td>0.7</td>
</tr>
<tr>
<td>chlorobenzene</td>
<td>26.0</td>
<td>26.7</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 3-2.
Separation results for the second experiment, in which the second peak was compressed.
Figure 3-5.
Gradient profile as determined by the program for producing the separation in the third experiment using "zone compression" for the first solute.
Figure 3-6.
The chromatograms for the first experiment (top), in which there was no "zone compression", and the third experiment (bottom), in which the first peak was compressed.
this elution profile and the chromatogram obtained in the first experiment for ease of comparison. It is obvious that the maximum absorption for the first peak increased when using the elution conditions presented in Figure 3-5. The peak height is 1.74 times than in the first experiment. The last capacity factor for this solute changed from 3.4 in the first experiment to 0.89 in this experiment. The actual retention times for the five solutes were 14.3, 19.5, 24.2, 26.1 and 28.8 minutes. The desired and actual retention times for this experiment are presented in Table 3-3. Using Equation 3-6 again, it can be shown that the absorption maximum for the first solute to elute in the third experiment should be twice the peak height of that in the first experiment.

The theoretical values calculated for the increases in peak height were both higher than the actual values. One possible reason for the deviation from theoretical prediction is that the concentration of the mobile phase reaching the end of the column was lower than expected due to mixing with adjacent mobile phase segments in the column. We were encouraged, however, since the trend was in the correct direction. In the second experiment, the k' changed from 5.1 to 0.99 and the peak was 0.93 times higher. In the third run, the k' changed from 3.4 to 0.74 and the peak was 0.74 times higher. This trend in experimental results
Desired and Actual Retention Times (min) in the Zone Compression Experiments

<table>
<thead>
<tr>
<th>Solute</th>
<th>Desired</th>
<th>Actual</th>
<th>Error(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzene</td>
<td>15.0</td>
<td>14.3</td>
<td>5.1</td>
</tr>
<tr>
<td>o-nitro-toluene</td>
<td>21.0</td>
<td>19.5</td>
<td>7.9</td>
</tr>
<tr>
<td>1-bromo-4-nitrobenzene</td>
<td>24.0</td>
<td>24.2</td>
<td>0.8</td>
</tr>
<tr>
<td>toluene</td>
<td>26.0</td>
<td>26.1</td>
<td>0.3</td>
</tr>
<tr>
<td>chlorobenzene</td>
<td>28.0</td>
<td>28.8</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Table 3-3.

Separation results for the third experiment, in which the first peak was compressed.
showed, that greater changes in k’ resulted in greater increases in peak height. This is in agreement with the theory that the UV detector signal is inversely proportional to \((1 + k')\) for the solute band as it elutes from the column.

Figure 3-7 gives the chromatogram obtained for the fourth experiment again with the results from the first experiment presented for ease of comparison. In this experiment, elution conditions were designed to force zone compression of the third peak (1-bromo-4-nitrobenzene). Figure 3-8 shows the profile determined by the program which results in the user requested retention times for the test solutes. It is clear from the chromatogram that the intensity of third peak was greatly increased. The final capacity factor for this solute changed from 7.0 in the first experiment to 1.15 in this experiment.

The following two examples will be used to demonstrate how this approach can be used to determine elution conditions which provide increased detection sensitivity for more of the solutes in a single chromatographic experiment. In this experiment, the goal was to determine an elution profile which produces retention times approximating those in the first experiment while providing increased detection sensitivity for the first, fourth and fifth peaks to elute from the column. Figure 3-9 gives the elution profile
Figure 3-7.
The chromatograms for the first experiment (top), in which there was no "zone compression", and the fourth experiment (bottom), in which the third peak was compressed.

a = benzene
b = o-nitrotoluene
c = 1-bromo-4-nitrobenezene
d = toluene
e = chlorobenzene

minutes
Figure 3-8.
Gradient profile as determined by the program for producing the separation in the fourth experiment using "zone compression" for the third peak.
Figure 3-9.
Gradient profile as determined by the program for producing the separation in the fifth experiment using "zone compression" for the first, fourth and fifth peaks.
determined by the program to provide the desired chromatographic results. The chromatograms obtained experimentally for the first and fifth experiments are given in Figure 3-10.

The next example in this series used elution conditions which were determined to provide approximately the same retention times as in the previous experiments but which resulted in an increased detection sensitivity for the third and fifth peaks of the test mixture. Figure 3-12 gives the chromatograms obtained for the first and the sixth experiments for these solutes when the elution conditions presented in Figure 3-11 were used.

3.6 Summary

As the examples demonstrate, this approach provides a powerful tool for increasing detection sensitivity and retaining efficient separation selectivity for HPLC elutions. This method could prove to be a very attractive strategy for trace analysis and for preparative separation.

Several potential problems exist, however, which may limit the routine use of this approach to methods development. First, a high level of performance and reliability are required of the HPLC instrumentation used in "zone compression" experiments. Even when using a high quality instrument it is possible that the isocratic
Figure 3-10.

The chromatograms for the first run (top), in which there was no "zone compression", and the fifth run (bottom), in which the first, fourth and fifth peaks were compressed.
Figure 3-11.
Gradient profile as determined by the program for producing the separation in the last experiment using "zone compression" for the third and fifth peaks.
Figure 3-12.
The chromatograms for the first experiment (top), in which there was no "zone compression", and the last experiment (bottom), in which the third and fifth peaks were compressed.

a = benzene
b = o-nitrotoluene
c = 1-bromo-4-nitrobenzene
d = toluene
e = chlorobenzene
retention data will change with time. Several steps can be taken to avoid this problem, including:

1. Completely degassing the solvent before starting the experiment
2. Obtaining the isocratic data for the highest strength mobile phase just prior to starting the gradient elution experiments. The reason for this is that the solutes move at their highest velocities under this condition and these data are very important for "zone compression" experiments. Small deviations will lead to large relative errors which could result in the short segment of mobile phase used for zone compression missing the band which is supposed to be compressed.

   It should be pointed out that even though the peaks in these examples clearly were greatly increased, it is difficult to quantify the improvement in detection sensitivity. This difficulty results from the fact that sudden large changes in the composition of the mobile phase affect the baseline of the chromatogram due to changes in refractive index. To measure the improvement, a chromatographic system which is capable of saving the elution data for the runs with and without the samples in the column is required. Using such a system, the background effect can be eliminated and the improvement in detection sensitivity can be calculated.
Additional research will be required to ascertain the actual increase in detection sensitivity provided by this approach under various elution conditions.
4.1 Introduction

An approach, along with a corresponding program, which provides computer-assisted methods development for multi-segment gradient elution experiments was described in Chapter 2. That approach was designed to assist the chromatographer in determining the retention time limits for each solute in a mixture and the elution conditions which provide the retention times desired for eluting each solute. One limitation of this approach to methods development is that the greater the number of solutes in the sample, the greater the number of gradient steps suggested by the program. Experiments showed that after the elution profiles determined by our program were used, there were differences between the actual chromatograms and the desired chromatograms.

One possible reason for the differences between predicted and actual retention times is that during the multi-segment elution process the gradient elution profile
is too complex to be accurately produced by the instrument. The gradient controller, which is a microprocessor used to control instrument operation, instructs the pump to generate different concentrations of the mobile phase at certain times. The pumps are sometimes required to provide a large concentration change within a very short period of time to elute the solutes at the user requested retention times, for example, changing the concentration linearly from 20 to 75 percent of B reservoir in 0.1 minutes. All calculations performed by the program, such as the capacity factors for each solute, the distances traveled by each solute and the retention times, are based on the assumption that the pump produces the profile given by the controller in an accurate and precise manner. It is often impossible for the pumps to exactly produce the profile requested by the methods development program, just as it is impossible for a person running fast to stop instantly even if ordered to do so.

Another potential reason for the difference between actual and predicted retention times is that, even if the mobile phase is accurately produced by the pumps, there are several mixing chambers which the solvent must pass through prior to reaching the solute in the column. Several of the sections have volumes where mixing between adjacent solvent zones can occur, including the gradient forming solenoid valves, associated mixing chambers, the pump heads, tubing
and column. Thus, it is reasonable to expect after travelling through these volumes the concentration of the mobile phase may differ from the initially generated profile due to the mixing of adjacent mobile phases. If an elution profile can be developed which has more gradual changes in mobile phase concentration between adjacent segments then it is expected that the actual elution profile will better agree with the theoretical elution profile.

Another, more practical problem is that the lifetime of a column will be shorter if it has to handle large rather than small concentration changes. This was seen in our experience as well as in that of others (41).

Thus, there are several reasons for determining if a simplified gradient profile which can produce similar chromatographic results can be found. It was postulated that if the number of elution segments could be reduced, the results would be improved. The reasons for this are as follows:

1. The accuracy requirements for the instrumentation may be lower, and the effect of the quality of the instrumentation on the resulting chromatogram will be reduced, which will enhance experimental reproducibility;
2. Column lifetime will be increased; and
3. The predicted retention times will be more accurate, because it is expected that there will be fewer
discrepancies between the theoretical and experimentally generated elution profile.

It is reasonable to ask therefore whether it is necessary to use a complex elution profile or can the number of gradient segments be reduced while still obtaining the chromatogram desired by the user?

The goal of this study was to answer the question posed above by designing an approach and corresponding program which would simplify a previously determined multi-segment gradient elution profile while retaining the desired retention time separations. The approach is based on determining an elution segment which provides essentially the same retention times for a given group of solutes using fewer elution segments. There are of number of possible elution profiles which can be used for simplifying a complex, multi-segment gradient profile. Using some of these simplified profiles may result in decreased selectivity or insufficient separation for compounds of interest. For this reason, the programs developed were designed to present to the user with the chromatographic results which will be obtained using the less complex elution profiles. The user is then allowed to decide which profile best suits the desired separation goals. Thus, the programs developed provide the user with several more options during the methods development process.
4.2 Program Description

To simplify gradient profile, the following procedure for reducing the number of elution steps was established. The time duration for the new gradient segment is fixed as the sum of the duration times for the old segments which it replaces. Thus, if two segments of five minute duration are to be replaced by a single gradient elution segment, then the new segment would have a duration of 10 minutes. The initial mobile phase conditions for the new segment are fixed at the final mobile phase conditions of the preceding gradient segment. For cases where there is no other unreduced segment before the new segment, the initial condition is the one which had previously been chosen by the user. Thus, the initial concentration of the mobile phase and the time length of the gradient segment which define the segment are known and the program needs only to determine the change in mobile phase concentration with time to define the new gradient segment.

There are several logical approaches to determine the slope of the new segment (i.e., change in % B per unit of time). It was decided to use a slope for the new segment which provides the same average mobile phase concentration as that experienced previously by the solute which eluted at the end of the final segment being replaced.

Figure 4-1 shows a gradient elution profile in which
Figure 4-1.
Gradient profile in which the second and third segments were replaced.
the independent variable is the gradient elution time and the dependent variable is the %B (the concentration of the mobile phase). The goal of this work was to find a single straight line segment which can be substitute for a number of connected line segments in a more complex elution profile previously determined.

The approach taken to determine the slope of the new segment will be presented both graphically and mathematically. Figure 4-2 shows the time axis divided into segments of the same length (0.1 minute segments were used in this study). Vertical lines are drawn from the end of each segment. These lines pass through the connected line segments that are to be replaced by the new segment. The intersection points on the segments to be replaced have coordinates of gradient time and %B. The origin is designated as the starting point of the first segment to be replaced. The general equation for the new segment is then given by the equation for a line which passes through the origin, which is:

\[ y = mx \]

were \( m \) is the slope of the segment to be determined.

For any point on the new line having coordinates, \((x_i, y_i)\), the distance from that point to the segment to be replaced with the same \( x \) coordinate, \( D \), is given by:

\[ D = mx_i - y_i \]  \hspace{1cm} (4-1)
ELUTION CONDITIONS

Figure 4-2.
Schematic diagram indicating the approach taken to determine the new segment which replaces the second and third segments. The time axis is divided into segments of the same length. Vertical lines are drawn from the end of each segment. These lines pass through the segments to be replaced and produce many intersection points.
The goal is to provide the same average mobile phase concentration as that experienced previously by the solute which eluted at the end of the final segment being replaced. It was decided that the sum of the distances from the points on one side of the new segment to the new segment should be equal to the sum of the distances from the points on the other side of the new segment to the new segment. This is demonstrated graphically in Figure 4-3. In mathematical terms, this relationship is given by the following equation:

\[ \sum_{i=1}^{n} (mx_i - y_i) = 0 \]  \hspace{1cm} (4-2)

which is can be converted to:

\[ \sum_{i=1}^{n} mx_i - \sum_{i=1}^{n} y_i = 0 \]  \hspace{1cm} (4-3)

Rearranging Equation 4-3 gives:

\[ m \sum_{i=1}^{n} x_i = \sum_{i=1}^{n} y_i \]  \hspace{1cm} (4-4)

Solving this equation for \( m \), the slope of the new gradient segment, gives:
Figure 4-3.
Schematic diagram demonstrating the sum of the distances of the points on one side of the new segment (represented by the dotted line) to the new segment should be equal to the sum of the distances from the points on the other side of the new segment to the new segment.
Thus, the slope of the new segment is equal to the sum of the \( y_i \)s of the points on the segments to be replaced divided by the sum of the gradient times of the points. The gradient elution time for the new segment is equal to the sum of the gradient elution times of those segments which are being replaced. The program uses the slope, the initial mobile phase concentration of the new segment and the gradient time to define the new segment which represents the new profile.

Once the initial concentration of the mobile phase, the gradient time and the slope of the new segment are determined, the question becomes how to incorporate this new segment into the remaining elution profile. Of particular concern is how to connect the new segment with the segment immediately following it. There are several possible approaches to solving this problem.

In this study, it was decided to allow the elution segment immediately following the new segment to have the same final concentration and time duration but to have a

\[
\sum_{j=1}^{n} y_j \quad \frac{m}{\sum_{j=1}^{n} x_j} \quad (4-5)
\]
starting concentration which is the same as the final concentration of the new segment. The remaining elution profile is thereby left unchanged from that developed previously. This is represented graphically in Figure 4-4.

An alternate approach for linking the new elution segment to the old profile is to keep the remaining segments exactly the same as before, introducing the new segment by forcing a very quick change in the mobile phase concentration at the end of the new segment to the same mobile phase concentration as at the start of the adjacent segment in the old elution profile. This approach has the potential for requiring a large change in mobile phase concentration in a short period of time. As discussed previously, there are many good reasons for avoiding elution conditions with sharp increases in solvent strength.

Once a simplified profile is determined, the program informs the user and calculates the retention time of each solute that will result if this elution profile is employed. The program also simulates and presents the chromatogram which will be produced using the new elution profile.

The process given above is repeated to reduce the number of elution segments even further. It reduces the elution steps from the first segment to the last segment, from just reducing one segment to the maximum number of the segments. A flow chart which demonstrates the logic and
Figure 4-4.
Schematic diagram demonstrating how to allow
the elution segment immediately following the
new segment to have the same final B% and time
duration but to have a starting B% which is the
same as the final B% if the new segment.
procedure used to simplify the elution profile is given in Figure 4-5.

Using the approach described, it is possible, at least in theory, for a user to reduce a complex elution profile containing several segments to a single linear segment. While such a reduction may not always be possible, the overall goal is still to provide the elution profile having the least complexity which provides chromatographic results that are suitable for the user.

4.3 Experimental

A. Instrumentation

The ternary gradient chromatographic system and the column that were employed in this experiment are identical to the ones described in Chapter 2. The dead volume of the column was 2.8 ml and the delay volume of the instrument was found to be 3.6 ml using acetone as the gradient marker with 90% v/v methanol/water mobile phase concentration. The flow rate was set at 1.0 ml/min.

B. Reagents

HPLC grade methanol and water (Fisher Scientific, Springfield, NJ) were used to prepare the mobile phases. Mobile phase A and B had concentrations of 30% and 85% v/v
Save the multi-segment gradient elution profile which would produce the desired retention times.

Calculate the sum of the gradient elution times, \( t \), for the elution segment to be replaced.

Calculate and save the coordinates of the B% and the gradient times for the points on the segments to be replaced when the increase in gradient time is 0.1 minutes, until the time is equal to \( t \).

Determine the new segment by:
- a. the beginning of this new segment as the point with coordinates of the initial B% and zero for the gradient time
- b. the gradient elution time for the new segment as equal to \( t \)
- c. the slope of this segment as equal to the sum of the B% of all the points divided by the sum of the gradient times of all the points.

Calculate the final B% of this segment by its slope and \( t \). Use this as the beginning of the following segment. For the following segment, keep the gradient time and the final B% the same as before. Save the new profile.

Recalculate the retention times for each solute using the new elution profile.

Present the results to the user for final verification.

Repeat this process for all possible combinations of replacing the multi-segment.

Figure 4-5.
Schematic diagram of the logic and procedure used to simplify the elution profile.
methanol/water, respectively. All mobile phases were filtered through a 0.45 μm nylon supported plain membrane filter and degassed using an ultrasonic bath and helium sparging before use.

The seven test solutes used for this study were benzene (ACS Spectranalyzed, Fisher Scientific Company, Springfield, NJ), 1-bromo-4-nitrobenzene (Practical grade, Eastman Kodak, Rochester, NY), toluene (Reagent grade, Fisher Scientific, Springfield, NJ), o-nitrotoluene (Reagent grade, Eastman Kodak, Rochester, NY), 2,4-dinitrotoluene (U.S. Army Cold Regions Research and Engineering Laboratory Reference Standard, Hanover, NH), diethylphthalate (Bakergrade, J.T. Baker, Phillipsburg, NJ) and methylbenzoate (Reagent grade, Fisher Scientific, Fair Lawn, NJ). Sample concentrations were approximately 0.001-0.005 v/v sample/methanol.

One of the reasons for choosing these test solutes was that the capacity factor vs. mobile phase composition curves for several of them intersect. If the capacity factor vs. mobile phase composition curves for two solutes cross, then both co-elution and reversal in elution order are possible for these compounds. The capacity factor vs. mobile phase composition relationships for the test solutes are given in Figure 4-6.
A plot of capacity factor vs. mobile phase composition (%B) for the seven solutes.

1 = 2,4-dinitrotoluene
2 = benzene
3 = methylbenzoate
4 = o-nitrotoluene
5 = 1-bromo-4-nitrobenzene
6 = toluene
7 = diethylphthalate
4.4 Results and Discussion

Several examples will be provided to demonstrate how the approach developed can be used to simplify a predetermined multi-segment gradient elution profile.

The goal of the first experiment was to determine a multi-segment gradient elution profile which completely separated the test solutes in less than 35 minutes. Using the approach to computer-assisted methods development previously developed and described in Chapter 2, a seven step elution profile which separated the test solutes was determined. The retention times of the seven solutes were chosen by the user after being provided with a range of possible retention times for each solute. The chosen retention times for the seven solutes were: 20.0, 21.0, 22.5, 23.1, 28.0, 32.0 and 34.0 minutes. The program determined the elution profile which would produce these results which is given in Figure 4-7a. The actual chromatogram obtained using this elution profile is presented next the profile. The actual and user desired retention times are presented in Table 4-1 for comparison. The percent deviation between the actual and desired retention times was less than 2.3% for this experiment.

Once the above elution conditions were established, the approach described above was used to simplify the elution profile by reducing the number of elution segments.
Figure 4-7.
The gradient profiles and obtained chromatograms in which
a. without simplifying.
b. one segment was reduced.
c. two segments were reduced.
d. three segments were reduced.
e. four segments were reduced.
f. five segments were reduced.
Calculated and Actual Retention Times (min) in the Simplifying Multi-segment Gradient Profiles Experiments

<table>
<thead>
<tr>
<th>Solute</th>
<th>Calculated</th>
<th>Actual</th>
<th>Error(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzene</td>
<td>20.0</td>
<td>19.7</td>
<td>1.5</td>
</tr>
<tr>
<td>2,4-dinitrotoluene</td>
<td>21.0</td>
<td>20.6</td>
<td>1.7</td>
</tr>
<tr>
<td>methylbenzoate</td>
<td>22.5</td>
<td>22.3</td>
<td>1.0</td>
</tr>
<tr>
<td>o-nitrotoluene</td>
<td>23.1</td>
<td>23.6</td>
<td>2.2</td>
</tr>
<tr>
<td>1-bromo-4-nitrobenzene</td>
<td>28.0</td>
<td>27.8</td>
<td>0.6</td>
</tr>
<tr>
<td>toluene</td>
<td>32.0</td>
<td>31.8</td>
<td>0.7</td>
</tr>
<tr>
<td>chlorobenzene</td>
<td>34.0</td>
<td>33.2</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Table 4-1.
Separation results for the first experiment without simplifying gradient profile.
The program presented many usable profiles and indicated the predicted retention times. Based on the simulated chromatograms displayed, some of the results were close to the chromatogram obtained in the first experiment and satisfied the requirement for no coelution and completion in less than 35 minutes. By using these profiles the actual chromatograms obtained were very close to the initially predicted chromatograms. Figure 4-7 f shows the profile, in which five segments were reduced, and the actual chromatogram produced using this profile. After a two-segment instead of a seven-segment elution profile was used, the seven solutes were separated completely in 34.2 minutes. The predicted retention times for the seven solutes were 19.9, 21.2, 24.6, 25.8, 29.5, 32.9 and 34.2 minutes. From Table 4-2, it is clear that the differences between the calculated and actual retention times were smaller for the two-segment elution than for the seven-segment elution experiment. Both elution profiles had the same gradient elution times: 27.63 minutes.

Figure 4-7 b, c, d and e show the elution profiles for reducing one, two, three and four steps in the actual chromatograms produced using these profiles, respectively. The separation results were very close to the result obtained in the first experiment, in which more elution segments were required.
Calculated and Actual Retention Times (min) in the Simplifying Multi-segment Gradient Profiles Experiments

<table>
<thead>
<tr>
<th>Solute</th>
<th>Calculated</th>
<th>Actual</th>
<th>Error(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzene</td>
<td>19.9</td>
<td>19.7</td>
<td>0.8</td>
</tr>
<tr>
<td>2,4-dinitrotoluene</td>
<td>21.2</td>
<td>20.9</td>
<td>1.3</td>
</tr>
<tr>
<td>methylbenzoate</td>
<td>24.6</td>
<td>24.3</td>
<td>1.4</td>
</tr>
<tr>
<td>o-nitrotoluene</td>
<td>25.8</td>
<td>25.5</td>
<td>1.3</td>
</tr>
<tr>
<td>1-bromo-4-nitrobenzene</td>
<td>29.5</td>
<td>29.1</td>
<td>1.5</td>
</tr>
<tr>
<td>toluene</td>
<td>32.9</td>
<td>32.5</td>
<td>1.1</td>
</tr>
<tr>
<td>chlorobenzene</td>
<td>34.2</td>
<td>33.8</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Table 4-2.
Separation results for the second experiment, in which five segments were reduced.
Not all reducing profiles, however, satisfied the separation requirement. Figure 4-8 shows the elution profile for reducing six steps. Figure 4-9 shows the predicted and the actual chromatograms. The first pair of solutes (benzene and 2,4-dinitrotoluene) partially overlapped and the last two solutes (toluene and diethylphthalate) were coeluted. This was not desirable; but this result had been predicted by the computer before the experiment, and the user could decide whether or not to use it.

4.5 Summary

As these examples demonstrated, the approach developed provides the ability to simplify multi-segment gradient elution profiles. The key feature is to use less segments and to retain sufficient separations.

One drawback in using this method for finding a new gradient segment to replace several old segments is that the final mobile phase concentration of a new segment is usually different from the final mobile phase concentration of the segment which it is to replace. This difference affects the segment which follows the new segment as demonstrated in Figure 4-4. This is one reason for the differences between the desired and the actual retention times of solutes which are eluted by the profile using the new simplified segment.
ELUTION CONDITIONS

Figure 4-8.
Gradient profile in which six segments were reduced by the program for producing the separation given in Figure 4-20.
Figure 4-9.

Experimentally obtained chromatogram produced using the gradient elution conditions presented in Figure 4-19.
To improve this method the rest of the profile should be recalculated using the method described in Chapter 2.
CHAPTER 5

CONCLUSION

Due to the time and effort required for HPLC methods development, a great deal of interest has been generated in computer-assisted approaches for this task. For many of these approaches, the traditional role of the digital computer has been expanded from that of just simply performing numerical calculations to one of also manipulating facts and information. The goal of the work described here was to design and evaluate approaches for advancing the use of computer-assisted interactive methods development for determining complex multi-segment gradient elution profiles. The results demonstrate that the computer can be a powerful tool for interactively assisting the chromatographer in developing complex multi-segment gradient elution methods. Computer simulation can also play an important role by allowing the user to determine which multi-segment gradient elution profiles will provide useful chromatographic data.

The major features of the work are:

1. This approach was designed to inform the user of
the chromatographic results which are experimentally possible using multi-segment gradient elution profiles based on the data provided. Using this information, which cannot be easily determined experimentally, the user is then capable of deciding whether the level of separation which can be achieved is acceptable for the experiment being conducted. Once this decision is made, the user is also able to specify acceptable retention times for each of the solutes in the mixture.

2. The approach determines multi-segment elution profiles which will produce the requested chromatographic results and presents the predicted resulting chromatogram to the user.

3. The system allows the use of "zone compression" to increase detection sensitivity for specified solutes. The goal in this case is to retain the retention times previously established for the other solutes in the mixture.

4. A strategy was developed and tested for sequentially simplifying the multi-segment gradient elution profiles previously established, while retaining the desired separation.

There are two major advantages to the use of this approach in assisting with complex HPLC methods development processes:
1. The time required to arrive at adequate conditions to provide separations meeting the requirements of the user is reduced.

2. This approach to methods development provides the user with increased control for determining which conditions may produce the required results. The approach allows the user to determine, before performing the experiment, which elution conditions are likely to provide unacceptable results.

Factors which affect the accuracy and utility of this approach for HPLC methods development include:

1. The number of isocratic elution data which must be acquired for each solute. Though the minimum number of experimental isocratic data points required is two, a greater number of isocratic data points will generally provide more accurate retention prediction.

2. The complexity of the capacity factor vs. mobile phase composition relationship for each solute.

3. Experimental and instrumental instabilities, such as flow rate, gradient profile, mobile phase and stationary phase stability, temperature, etc..

Based on the encouraging results obtained using this approach for HPLC methods development, several additional investigations are warranted:

1. The continued improvement in the computational abilities
of the programs developed.

2. The development of approaches and logic sufficient to produce an expert system for determining multi-segment gradient elution profiles.

3. The investigation of other functions or options which would allow the generation of useable multi-segment gradient elution profiles in more efficient ways.

The results indicate that the approach developed could provide the computational method which will be the basis for future HPLC methods development expert systems.
LIST OF REFERENCES


